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A SYSTEM OF BACTERIOLOGY IN RELATION TO MEDICINE

VOLUME I

LONDON:
PUBLISHED BY HIS MAJESTY'S STATIONERY OFFICE
1930

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For this Volume Professor ARTHUR HARDEN has assisted in the editorship with regard to particular sections.

A SYSTEM OF BACTERIOLOGY IN RELATION TO MEDICINE

VOLUME I

BY

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GENERAL PREFACE

By The Rt. Hon. the Earl of Balfour, K.G., O.M., F.R.S.

I count myself most fortunate in being privileged, as Chairman of the Medical Research Council, to introduce to the scientific world (if only in the capacity of Master of Ceremonies) this most notable record of bacteriological investigations already accomplished, this valuable aid to discoveries still to come. The English-speaking peoples have, I believe, done their full share of the international labours devoted during the last fifty years to developing Pasteur's fundamental discoveries and Lister's epoch-making application of them to medicine. But no comprehensive treatise embodying the results of modern discoveries exists in the English language comparable to these nine volumes now in process of publication with the assistance and by the authority of the Medical Research Council.

Such an undertaking requires co-operation on the largest scale. No single enquirer, no single school of enquirers could deal unaided with the whole field of bacteriological study. This is not one of the subjects in which the progress of science has tended to simplification. It might be thought that our changing views of physical reality, as these have been developed during the last thirty or forty years by mathematicians and physicists, present a typical example of how science grows. Electrons and Protons, non-Euclidean space, and all the mysteries of modern Relativity, however hard of comprehension they may seem to plain men (like myself), do obviously provide us in spite of admitted difficulties with what is intrinsically a much simpler scheme of the material universe than the one which it is replacing. But if the progress of physics has (in a sense) simplified our conceptions of the vast and lifeless spaces of the world, the contemporary progress of biology has produced a revolution in our conceptions of exactly the opposite kind. To be sure this only touches that small corner of the universe where organic life seems possible. But as this small corner is our home, its newly realized complexity must needs be of the deepest interest.

Man, I suppose, has always concerned himself mainly with what lives. Friends and enemies of his own kind, animals which he eats, or by which he is eaten, plants which he consumes as food or uses as material—I say nothing of gods, ghosts and imaginary monsters—have mainly filled his thoughts and governed his behaviour. They do so still. But the conception of our living environment has in quite recent years suffered a most startling extension. We now know that among the organisms which crowd the thin terrestrial crust of land, air and water to which they and we are rigidly confined, incomparably the most numerous are invisible to our

unaided eyes. Man, for good or evil, has lived in closest association with these unseen multitudes through all the ages, yet never before modern times has he suspected their existence, nor till a period still within living memory has he counted them among the most formidable, as well as the most necessary, of the natural agencies with which he has to reckon.

Here then is a field of research, new and limitless, with a double claim on the attention of its explorers. It appeals, as the unknown always appeals, to enlightened curiosity. But it does much more than this. The knowledge which it is in process of giving us turns out to be directly and intimately connected with every phase of human interest and activity, with life and death, with health, happiness and efficiency, with plague, pestilence and famine, with the fertility of our fields and the vigour of our live stock.

Now, it is not merely the immense importance of these interests which has to be borne in mind when we are considering the value of a work like the present, it is their embarrassing variety, and the different points of view which have to be studied. Who in these days can hope to advance his subject unless he be ready to work as a specialist? How can any specialist acquire the breadth of vision needed for great discoveries? Such practical difficulties present themselves in all important spheres of research, but in none, I imagine, more evidently than in bacteriology—a rather special case.

The case is special owing to the fact that though bacteria are the subject matter of bacteriology they do not, in and for themselves, constitute its main interest. It is their relations to higher organisms which chiefly engross our attention; and to these relations is due the fact that bacteriology is so intimately concerned with such scientific arts as preventive medicine, clinical practice, veterinary work, sanitary engineering, agriculture; and with such complicated but all-important problems as infection, and the various modes in which bacteria are transferred from one host to another.

If this view of bacteriological problems has any resemblance to the facts it clearly indicates the pressing need for some such work as the one which I have now the honour of introducing. For it appears that, though all these problems are connected, they must be attacked from many different centres by investigators labouring in far-separated fields, who have not received the same training and do not employ the same techniques.

How is this unity of effort to be fully maintained by those who of necessity follow paths so widely separated? This is a question which I cannot answer; but of this at least I am sure, that the nine volumes of this work will enormously facilitate the task. They will make it easy for enquirers to find out what has been already accomplished, and to consult, if they desire it, the original sources on which the work is based. They are a gift from specialists to specialists. No investigator, however narrow may be his intensive study of one aspect of one problem, need henceforth feel that in cultivating this small part he must lose his vision

of the whole; and no one endeavouring to the best of his ability to form some general picture of the whole need doubt that with the aid of this System of Bacteriology he will succeed in his efforts. If these happy results are attained, the Medical Research Council and their brilliant army of collaborators will have every reason to feel that they have made a great contribution to contemporary science.

9th March, 1929. *Chairman, Medical Research Council.*

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CHAPTER I. HISTORY OF BACTERIOLOGY.

By William Bulloch (London Hospital).

Ancient Doctrines of Epidemic Disease down to Fracastoro.

In modern times it has been demonstrated beyond all doubt that a large number of diseases are brought about by the entry into the body of microscopic living agents. Such infective diseases affect individuals or collections of individuals. At all periods of written history there are records of epidemics and epizootics, but it must be recognized that an exact diagnosis of many of the ancient plagues is not possible to-day. The words λοιμὸς, pestis, pestilentia, were applied by the ancients to any epidemic disease irrespective of its nature or symptoms. The idea of contagion is an old one. The Jews, for example, believed in the risk of contact with disease as is clearly indicated in the Bible (Leviticus XIV ; Numbers, XII, 15), but all authorities are agreed that there is not the slightest reference to contagion in the Hippocratic treatises. It is strange indeed that, among the Greek and Roman writers, this idea came not from the medical but from the lay writers. In his history of the great Athenian plague (430 B.C.), Thucydides tells us that the disease touched or attacked (ἥψατω) the men of the Piræus, and it was said that the Peloponnesians had actually thrown poisons (φάρμακα) into the wells. The epidemic at the siege of Potidæa (Thucyd., Chapter LVIII) was also considered to be propagated by contact. References to similar contagious epidemics also occur in the writings of other historians such as Dionysius of Halicarnassus, Diodorus Siculus, Dion Cassius, Appian, Livy, and even in the poets, Lucretius and Virgil.

Although in the minds of these writers there was a vague idea of communication of disease by touch, epidemic diseases were referred chiefly to supernatural agencies and especially to divine judgments which were to be counteracted by sacrifices and lustrations. Thus in the Homeric poems (Iliad I, V. 50–52), Apollo was the deity who with his darts inflicted epidemic disease on the army before Troy, and the malady could only be overcome through the supplications of Chryses (Ganszyniec, 1923).

This theurgical theory of pestilence lasted for centuries, but was gradually displaced by the idea that epidemic diseases arise from natural and especially cosmo-telluric causes such as comets, earthquakes, inundations and changes in the air, which was considered to be vitiated by miasms (μιασμα, a stain). The modification of the atmosphere as a result of seasons and climate was a favourite theme of Hippocrates.

Just as the seasons succeed each other, he believed that the body underwent seasonal changes which exerted an influence on the diseases of the period, and from this developed the doctrine of ' pathological constitutions ', an idea held for ages and supported by Sydenham as late as the seventeenth century.

The late Greek and Roman medical writers contented themselves with verbose commentaries on the Hippocratic ideas, and even the Arabian physicians of the East and West, although they added to our knowledge of the symptoms and differential diagnosis of epidemic diseases, did not advance beyond the Hippocratic and Galenical doctrines of ætiology (Seidel, 1913). During the middle ages, however, the doctrines of infection and contagion began to emerge more clearly.

In *The Decameron*, Boccaccio, for instance, referred succinctly to infection, directly or even by touching the person or the clothing of the sick. Epidemics of small-pox, typhus fever, the black death, the English sweats from 1485 to 1551, and the pandemic of syphilis in the sixteenth century gave abundant scope for observation and reflection. Alessandro Benedetti (1493) who taught that disease may be contracted by touch and that the morbific principle is imbibed and conserved in articles used by the sick, inculcated the necessity in purifying the clothing. The merit of placing these suggestions on a wider basis we owe to Girolamo Fracastoro (1483–1553) of Verona. To explain contagion he postulated the existence of seeds of disease—*seminaria prima*—which were capable of being propagated from one individual to another. In his opinion, contagion was transmissible (1) by direct contact alone, (2) by contact and fomites and (3) at a distance. The word ' *fomes* ' was used by the classical writers to denote touchstone or tinder, but by Fracastoro in a special sense for such things as clothing, furniture and other objects (' *fomitem apello vestes, ligna et ejusmodi* ') which non-corruptible in themselves could conserve the seeds of contagion and thus infect. Fracastoro likened contagion by direct contact to the corruption that sets in when grapes or apples come into contact with each other. He supposed that the ' *seminaria* ' of disease propagate and generate until the whole mass is affected. Some have supposed that Fracastoro considered that the ' *seminaria* ' of disease were necessarily living and his writings may be interpreted in this sense. On the other hand, he compared the idea of disease propagation with that of *halitus* or exhalation, for just as the onion can cause the eyes to water, so a *halitus* from a contagious ophthalmia can produce a similar state in another individual at a distance. The remaining portions of Fracastoro's work are given up to the accurate description of the great Italian epidemics of typhus fever (1505 and 1528), plague, rabies, syphilis and the general therapy of contagions. He touched on the immunity which ensues after an attack of measles or smallpox. Altogether, Fracastoro's book on contagion must be reckoned for the period as a great, if isolated, landmark in the doctrine of contagion. During his lifetime and for many years it was held in the highest repute, but its

importance was gradually lost sight of by the end of the sixteenth century, and all that he had achieved had to be rediscovered in the nineteenth century.

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Contagium animatum. Discoveries of Leeuwenhoek.

The theoretical speculations of Fracastoro and his immediate successors suggesting the existence of seeds of disease led to the doctrine of *contagium vivum*, which was soon to find an objective basis through the invention of the microscope about the end of the sixteenth century (Harting, 1848–54). By means of this instrument an undreamt-of world of invisible living things was now made visible.

The early microscopists of the seventeenth century concentrated their attention chiefly on small animals, and to this period belongs the discovery of the itch mite by Hauptmann (1657) and Ettmüller (1682). The real

discoverer of living things of microscopic dimensions was, however, the great Dutch microscopist, Anthony van Leeuwenhoek of Delft (1632–1723). This wonderful man of unparalleled industry was not educated in the ordinary sense, and spoke and wrote no language except his mother-tongue. He made his own microscopes, which were all of the single or simple class—in fact, merely magnifying glasses. Some have believed that his lenses were melted glass globules, but Henry Baker (1753), who had in his hands the cabinet of 26 microscopes bequeathed by Leeuwenhoek to the Royal Society, states that they were ground surfaces. In the said cabinet, now lost, the magnifying powers of the lenses ranged from 40 to 160 linear diameters. It is known, however, that this was not the limit, but that Leeuwenhoek had made lenses magnifying up to 300 diameters. The appearance of one of Leeuwenhoek's microscopes is shown in Fig. 1.

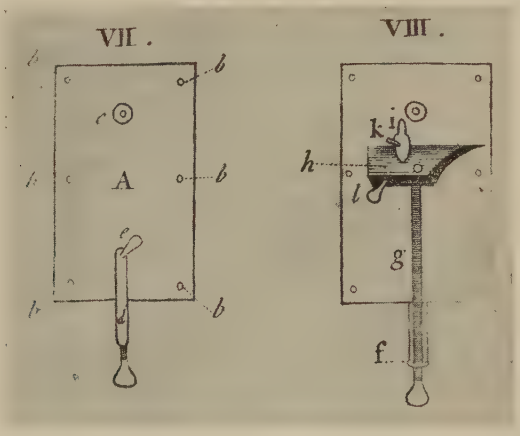

FIG. 1.—One form of Leeuwenhoek's microscope. From H. Baker (1764).

It consisted essentially of two thin silver plates fastened together by rivets and between the plates a small lens was let in at *c*. A limb *d* of silver was attached above to the front of the microscope by a screw *e*, while below it projected behind and was traversed by a long fine-threaded screw *g* which turned in and raised or lowered a stage *h* on which was a pin *i* for attachment to the object to be examined. The pin, *i*, was capable of being turned by a small handle or projection, *k*, and the position of the stage with the pin could be regulated by a small screw, *l*, which passing through the stage horizontally and bearing on the back of the instrument could thrust it forward. This was the type of microscope ordinarily used by Leeuwenhoek, but he employed others for special purposes. He is known to have made about 400 separate microscopes. He estimated magnification by comparing the objects under examination to such things as grains of sand, hairs and human blood corpuscles.

Leeuwenhoek probably began his microscopic observations about 1670, and it was in 1673, when he was 41 years old, that his younger fellow

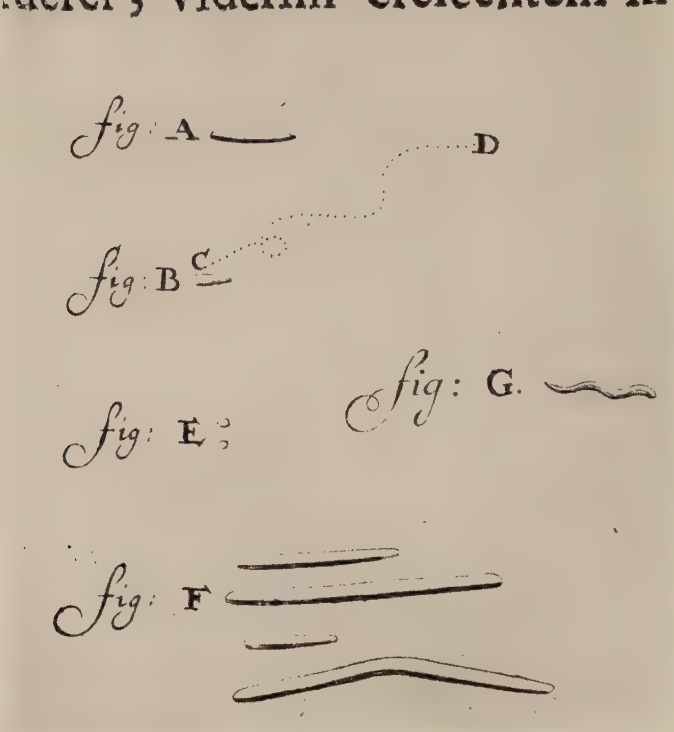

FIG. 2.—Bacteria figured by Leeuwenhoek. From Leeuwenhoek (1695).

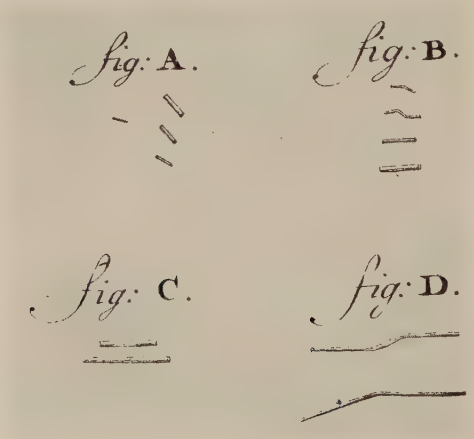

FIG. 3.—Bacteria figured by Leeuwenhoek. From Leeuwenhoek (1695).

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townsman, Reinier de Graaf (1641–73), introduced his name to the Royal Society, and from this time till his death half a century later, he was one of the Society's most valued contributors; he was elected a Fellow of the Royal Society in 1680, N.S. Leeuwenhoek's principal discoveries are given in a series of nearly 200 letters written to the Royal Society and still preserved in its library. In one of these letters, dated 9th October, 1676, he tells us that it was in 1675 that he discovered living creatures in rain water which had stood for a few days in a new earthen pot. He described four sorts of animals of which one was undoubtedly a *Vorticella*. The fourth kind was so small that he could not give a figure to it at all, but 'they were', he says, 'one thousand times smaller than the eye of a big louse'. He also found microscopic creatures in water from the Maas, from a well in the courtyard of his house in Delft, and in sea water which a friend had brought him from Scheveningen. He also gave a long account of an incredible number of animalcules which he discerned in infusions of peppercorns in rain water. According to our present knowledge, the majority of the creatures described by Leeuwenhoek in 1676 were protozoa, although the most minute were probably bacteria. In a letter (No. 32) dated 14th June, 1680, he related that yeast was composed of globules of matter floating in a clear medium, and he describes the continued existence of animalcules and bacteria living under partial anaerobic conditions. If there is doubt as to the bacterial nature of some of the living creatures described by Leeuwenhoek in 1676, there can be none concerning those that he described in 1683. In his 39th letter (dated 17th September, 1683) he clearly described bacteria which he had found on his own teeth. The biggest sort had the shape of A (Fig. 2), their motion was strong and nimble and they darted through the water or saliva as a jack or a pike darts through water. The second kind in the shape of B spun about like a top and took a course sometimes on one side as is shown in C and D. In the third sort, he says, he could not well distinguish the figure, for sometimes it appeared to be oval, at other times a circle. They were so small that they appeared no bigger than E and therewithal so swift that 'I can compare them to nothing better than a swarm of flies or gnats flying or turning among one another in a small space'. Besides these animals there were a great many streaks or threads of different lengths, but like thickness, lying confusedly together, some bent, some straight as at F. These had no motion in them. Leeuwenhoek found the same 'animals' on the teeth of two women and a child eight years old. The substance on the teeth of an old man who had lived soberly had a great many living creatures swimming more nimbly than he had previously observed. The biggest sort were numerous and as they moved bent themselves like G. Leeuwenhoek gargled and washed out his mouth with strong wine-vinegar but still found the animalcules on his teeth. He estimated that the number of them on a man's teeth 'exceeded the number of men in a kingdom'.

This is the first account of undoubted bacteria and the description given is largely in Leeuwenhoek's own words. It is remarkable that with his primitive lenses he should have seen so much, because it is quite clear that he describes and figures the chief morphological types of bacteria that we know to-day, viz. : cocci, bacteria and bacilli, and spirilla or spirochætæ. Leeuwenhoek returned to the subject in his 75th letter (dated Delft, Oct. 1, 1692). This is given in Latin in his 'Arcana naturæ detecta' (1695, p. 335), and it is from this I quote. He tells us that he had been unable to confirm his observations of 1683 on the dental animalcules, and he concluded that it was probably due to his habit of drinking excessively hot coffee. But having carefully cleansed his mouth and taken material from his molar teeth by the aid of a mirror he was overjoyed to find the dental animalcules again, and they were so active that a particle of matter under examination seemed alive with them. He tried to measure the smallest of them in comparison with a grain of sand such as is used for polishing tin, and he was forced to admit that the sand grain was more than one million times the size of one of the animalcules. In addition to the smallest animalcules of rounded form he described and figured four types (Fig. 3), which can be clearly identified as bacteria. Some of the animalcules had bodies five or six times longer than broad, and the bodies were equally thick all through so that he could not distinguish an anterior from a posterior end. Their motion was very slow and seemed to be by a process of bending. Their appearance is figured in A. A second class, B, had the same length as A, but were rather longer, and moved with great flexion of their bodies. 'By these bendings', says Leeuwenhoek, 'they started so swift a movement, sometimes swimming forward, sometimes backward and especially rolling themselves round on their long axis, and not without wonder and delight did I see them again, and that all the more because for some years I have not been able to detect them. The swift movement of their bodies moving about among the very minute animalcules resembled a butterfly flying about in a swarm of gnats which were driven away by its wings'. There were other animalcules, C, about the same thickness but of divers lengths. Their motion was so slow that they appeared not to move at all, but, says Leeuwenhoek, 'when I fixed unwearied eyes on them I saw their bodies bending very slowly, with languid motion, making little progress'. The fourth type, D, were of the same thickness as C, but much longer, and occasionally two of the same kind close together appeared progressing together through the liquid. This type was in great profusion, and, while some were straight, others were bent, but the larger they were the less could Leeuwenhoek detect vitality or movement in them, yet he felt certain they were living or had been alive while they were in the mouth adherent to the teeth.

Leeuwenhoek frequently refers to the great pleasure he experienced in studying the minute creatures with his lenses and he affectionately speaks of them as 'beasties' (beesjes) or 'little creatures' (cleijne

Schepsels). He was the first bacteriologist and the first protozoologist, and, as Dobell (1923) justly remarks, he ' created bacteriology and protozoology out of nothing '.

It was a long time before Leeuwenhoek's observations on bacteria were confirmed, and this must be put down to the imperfect microscopes used by others. The larger animalcules were naturally much more easily observed, and thus it was that most of the early works on microscopic animals refer to these rather than to the bacterial forms which Leeuwenhoek alone had clearly seen. The animalcule period confirming Leeuwenhoek began with L. Joblot (1718), and was continued by many other workers during the eighteenth century. In 1771, 1773–4 and 1786 appeared the great works of O. F. Müller, in which an attempt was made to classify not only animalcules but also bacterial forms. A detailed account of the works of all the early writers on animalcules has been given by Bütschli (1887–9).

The observations of Leeuwenhoek contributed to the revival of the ancient belief that certain diseases might be caused by invisible living agents. This idea was concretely stated by Varro (116–26 B.C.) and Columella (*circa* 60 B.C.), two of the early writers on husbandry. In the seventeenth century Athanasius Kircher (1658), C. Lange (1659, 1688) and Lancisi (1718) were ardent supporters of the doctrine of *contagium animatum*. The theories of these writers were, however, devoid of objective basis and were not taken seriously. In fact they appear to have been lampooned in the ' Systême d'un medicin Anglois ' published over the initials M.A.C.D. (1726) and purporting to describe the animalcular causes of disease. Eighty-seven of the supposed creatures are figured and named after the different pathological or even physiological conditions in which they were alleged to be found. Names like ' *apopleptiques* ', ' *assoupissans* ', ' *astmatiquans* ', ' *bubonistes* ', ' *erectifs* ', ' *dentaires* ', ' *gonhorriques* ', ' *migrainistes* ', ' *canceriques* ', ' *sciatiquans* ', ' *vapeuristes* ' will explain themselves. C. Singer (1911) has drawn attention to the existence of a neglected but remarkable work of this period by one Benjamin Marten (1720) concerning whom nothing is known. He came uncannily near the doctrines of infective disease held to-day, and suggested an ætiology of consumption which coincides exactly with the views propounded by the majority of authorities now. During his time, however, there was no support for his views. Even Linné, famous man that he was, doubted the value of microscopy. He did not even believe that there were such things as spermatozoa, for he passed the statement in Ramström's thesis (1759) that what had been taken for spermatozoa were only motile particles whose movement depended solely on the heat of the spermatic fluid. Nyander (1757), another of Linné's pupils, contended that the cheese mite and the itch mite were one and the same, and that dysentery, variola, measles, plague and syphilis are all caused by mites. Linné found himself unable to classify the animalcules revealed by Leeuwenhoek and Joblot. In his ' Systema naturæ ' as late as 1767 he grouped them under ' Vermes '

and in a class which he called ' Chaos ' with six species, the last of which was ' Chaos infusorium '. As an appendage he added :

α. Febrium exanthematicarum contagium ?
β. Febrium exacterbantium causa ?
γ. Siphilitidis virus humidum ?
δ. Spermatici vermiculi, Leeuwenh. ?
ε. Aethereus nimbus mense florescentiæ suspensus ?
ζ. Fermenti putredinisque septicum, Munch. ?

These he regarded as small molecules which lie hidden and perchance belong to ' Chaos '.

It is surprising to find Linné, 30 years after Leeuwenhoek's death, placing in the same class spermatozoa and the ' ethereal clouds in the time of flowering '. That Linné's views, however, were not accepted by all is seen in the book of M. A. Plenciz (1762), who regarded the seeds of contagion as air borne and as developing into countless animalcules.

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Fermentation.

The phenomena of fermentation as it occurs in the production of bread and wine must have been observed and excited wonderment at all times, but it was only in the nineteenth century that the process began to be understood, and the knowledge gained played an important part in the development of bacteriology. It was chiefly from the English physician Thomas Willis (1659) that the conception of fermentation as an internal commotion of the particles of the fermentable substance took place, and this idea recurs again and again in the chemical literature of succeeding years. From the examination of Appert's preserves, J. L. Gay-Lussac (1810) concluded that oxygen is necessary to start fermentation, but not for its continuance. Appert (1810) was a retired confectioner and distiller who wrote a small book on the preservation of food-stuffs. In his process the material was placed in clean well-corked bottles which were then raised to the boiling-point for varying periods according to the nature of the viands.

In the first third of the nineteenth century a new direction was given to research on fermentation by the discovery that yeast is a living organism. The pioneers in this work were Baron Charles Cagniard-Latour (1836), Theodor Schwann (1837) and Friedrich Kützing (1837). Before their time, yeast was regarded as a chemical substance of unknown chemical constitution. That yeast has a globular structure was discovered by Leeuwenhoek (1680) and Desmazières (1826). Cagniard-Latour (1777–1855) in a sitting of the Société philomathique of Paris (June 18, 1836) expressed the conviction that yeast was a living thing of vegetable nature, and in 1837 he suggested that fermentation was probably the result of the development of the yeast plant in saccharine solutions with the consequent production of alcohol and the evolution of carbon dioxide. He described the reproduction of the yeast globules by a process of budding. Cagniard-Latour (1838) published a general account of his researches on fermentation, after which he wrote nothing more on the subject.

The true nature of yeast was also recognized independently by Schwann (1837) who regarded it as ' without doubt a plant '. He clearly stated the connection between the growth of the yeast plant and the occurrence of fermentation. He spoke of yeast as the ' Zuckerpilz ' from which the term ' Saccharomyces ' took its origin. He showed also, that in addition to yeast, fermentation in a sugar solution requires the presence of a nitrogenous substance. His view was that the yeast plant drew its food supply from the sugar and the azotic substance and left behind alcohol and other compounds. In his famous ' Mikroskopische Untersuchungen ' (1839) Schwann gave his reasons for regarding the yeast globules as fungi. These reasons were that their form was that of fungi and they grew like fungi. That they caused the fermentation followed, in Schwann's opinion, (1) from the constancy of their occurrence during the process,

(2) from the cessation of fermentation under any influence by which they are known to be destroyed, and (3) because the element which excites fermentation must be a substance which is regenerated and increased by the process itself—a phenomenon met with only in the case of living organisms.

Kützing (1837) also gave a clear account and an excellent illustration of the yeast cell. He made no reference, however, to its mode of reproduction, and went astray in attributing to it a relatively pleomorphic cycle, beginning with the ordinary oval globule and finishing with a full-blown mould. The work of Cagniard-Latour and Schwann was confirmed by Quevenne (1838) and by Turpin (1838) in an elaborate monograph illustrated with excellent plates (1840).

The biological theory of fermentation did not commend itself to the majority of the scientists of the day. The reason is not far to seek. In the first third of last century chemistry had made great strides, especially under the influence of the Swede, J. J. Berzelius (1779–1848), whose pupil, F. Wöhler (1800–82), had shown that it was possible to prepare from inorganic materials products which, it had been previously believed, could only be found in the living body. His synthesis of urea dates from 1828. Justus Liebig (1803–73), a friend of Berzelius, was also imbued with the new chemical progress, and throughout his life strove to impart a purely chemical character to every so-called vital process. It is not surprising that the trio of friends, Berzelius, Wöhler and Liebig, should have arrayed themselves against what they regarded as the erroneous teaching of Cagniard-Latour, Schwann and Kützing. The campaign was opened by an opprobrious attack on Schwann by Berzelius (1839) who also contemptuously dismissed Kützing's communication with the remark that it possibly possessed some value microscopically but none scientifically. Berzelius developed the view that fermentation was an example of the ' catalytic principle ' which he himself had suggested in 1836.

Under the title ' The riddle of vinous fermentation solved ' a vulgar skit on the supposed nature of yeast appeared anonymously in 1839. It is now known (see Wöhler, 1901) that the authors were none other than Wöhler and Liebig. It was stated that when yeast globules were placed in a sugar solution they gave birth to unknown animalcules resembling in appearance Beinsdorff's distilling apparatus (a most complicated piece of mechanism). From the anus of the animalcule a great stream of alcohol issued, while from the enormous genitals bubbles of carbon dioxide were given off.

Liebig did not, however, fight only with the weapon of ridicule but made a more serious attack in a paper (1839) in which he formulated his views on fermentation. These views he maintained more or less unchanged until his death. He attributed the processes of decomposition, eremacausis, putrefaction and fermentation to the chemical instability of certain substances which could communicate their instability to other substances in succession. The unstable substances he called ferments, and they were

supposed to arise as a result of some change in vegetable solutions exposed to air, and to continue the fermentation, once started, in the absence of air. Liebig admitted the globular characters of yeast, but neither he nor his pupil Schlossberger (1844) believed that there was anything living about it.

Mitscherlich (1841) made an interesting experiment which showed that yeast globules will not traverse filter papers. A glass tube, the bottom of which was closed by filter paper, was suspended in a jar containing sugar solution. The tube being filled with the same solution, a trace of yeast was placed in it. Fermentation occurred in the tube but not in the jar. Although, in Mitscherlich's opinion yeast was essential for fermentation it acted not as a vital agent, but merely by its presence, and in this respect he supported the catalytic theory of Berzelius. Blondeau (1847) examined several different types of fermentation and expressed the opinion that each was produced by a different fungus.

As a result of all these and many other researches a great deal of knowledge had accumulated on the subject of fermentation. The extent of this knowledge may be gauged by the perusal of the large work by Balling (1845). This brings us down to the middle of last century, when fresh advances were made through the genius and industry of Pasteur (1822–95). He was not the creator of the doctrine that fermentation is due to the activity of living microscopic organisms, but he built on more solid foundations than his predecessors, who in some degree failed to leave their impress on the scientific thought of their day. The time was not ripe for their efforts, and there is much truth in the Franklands' remark that ' Pasteur was not only a *savant* content to seek the truth and find it, but that when he had in any matter succeeded in convincing himself he was impelled with almost a fanatic's zeal to force his conviction on the world, nor did he put up his sword until every redoubt of unbelief had been taken, every opponent converted or slain '.

Pasteur did a great deal and wrote a great deal, and much has been written about his work and the man himself. His name will recur frequently in these pages, for there was no department of bacteriology that he did not enrich. At present, however, I confine myself to his efforts to unravel the problems of fermentation, a subject with which his name will for ever be associated. Pasteur was a chemist, and his early work (1849), on the constitution of tartaric acid, had already made him famous in the scientific world. It was not until 1854 that he began to interest himself in fermentation, and in 1857 he published his first communication on the lactic fermentation. Tracing the progress of this well-recognized change he noticed a greyish substance as a deposit or lying on the surface of the fluids undergoing the lactic change. Under the microscope the deposit was found to ' consist of minute globules, or very short pieces single or in masses '. Taking a trace of the grey deposit and placing it in a solution of sugar containing some chalk, Pasteur saw active lactic fermentation taking place and the process could be repeated with the same result.

Each time the deposit showed the same features. He argued that the new ' yeast ', as he called it, was organized and living, and that its action on the sugar was correlated with its organization and development. In 1859 he showed that in a solution containing sugar, ammonium phosphate and chalk a cloudiness appeared and gas was evolved. As the fermentation proceeded the ammonia disappeared, phosphates and calcium salts were dissolved, lactate of calcium was formed and the lactic ' yeast ' settled to the bottom of the flask. He showed that in all probability the lactic ferment came from the air, for when he used sterilized solutions of the various ingredients and allowed only heated air to enter, neither lactic fermentation, nor lactic acid, nor ' yeasts ' appeared. The fluid remained barren. The essential points of all Pasteur's subsequent work on different fermentations are epitomized in his short paper on lactic fermentation. We have here the recognition of the necessity of finding the optimal condition for the fermentation, and from this the determination of the minimal requirements of the ferment. There is the examination of the microscopic organisms which develop during the fermentation in the simplest pabulum, and the demonstration of the constancy of their characters. Finally it is shown that a minimal trace of the ferment induces the fermentation.

By such methods Pasteur worked his way through a large number of different fermentations during the next twenty years (1857–77), and with a success which stamped him as a master in this line of enquiry. To record all that he found out has filled volumes. I can only indicate here some of the most important problems which he studied. His earliest work on alcoholic fermentation dates from 1857 and reached its height in his classical ' Mémoire sur la fermentation alcoolique ' (1860). In this, he revealed himself as totally opposed to the doctrines of Liebig, and as a staunch upholder of the view that yeast is living, and that during its vital activity it splits sugar into alcohol, carbon dioxide and other substances. In 1858 he made a remarkable discovery in connection with tartaric fermentation. He observed, if perhaps incompletely, the characters of the organism which ferments ammonium tartrate, and on transferring a trace of the ferment to a solution of ammonium racemate, which is optically inactive, he noted that the fermenting solution gradually acquired lævo-rotatory powers, until finally the right-handed salt disappeared and lævo-tartaric acid was left untouched. In 1861 he turned his attention to butyric fermentation and discovered that it proceeds in the absence of air. In the fermenting fluid he found cylindrical rod-shaped organisms and proved that they caused the fermentation. The organisms—he called them ' vibrios '—lived without oxygen. On examining under a cover glass a drop of fluid containing them, he was astonished to see that on the margin of the drop, where it was in contact with air, the vibrios had ceased to move, although in the centre they were still actively motile. Oxygen apparently was inimical to them, and he supported this view by showing that a stream of oxygen, passed through a fluid in active

butyric fermentation, impedes or stops it altogether. He discovered other micro-organisms that live without air, and established the idea of aerobic and anaerobic life, and was the first to employ these terms (1863).

In 1861 he began to publish papers on the acetic fermentation, and showed that it likewise is due to special organisms of the genus *Mycoderma*. These mycoderms were studied in greater detail in 1862, and his several researches on the subject appeared in 1864 and 1868 under the title ' Études sur le vinaigre '. In 1866 he published his famous ' Études sur le vin ', a work of more than 200 pages. In it he dealt with what he called ' diseases ' of wines, and showed that they were due to alien micro-organisms which invade the wine and lead to its deterioration. He also laid down the principles whereby the wine diseases could be minimized or abolished.

About 1865 Pasteur's researches on fermentation were interrupted, for he was sent to study the silkworm disease then ravaging France. This occupied him six years, and it was only in 1871 that he was able to take up fermentation again and especially in connection with the production of beer. His ' Études sur la bière ', a work of nearly 400 pages, was published in 1876, and deals with many points which were of fundamental importance in the development of bacteriology as a science. For example, he investigated the question of the presence of bacteria in normal tissues, and the transformation of one species into another. The book is also interesting as containing his mature views on fermentation in general. In his opinion fermentation is essentially the result of life without oxygen, ' C'est la vie sans l'air, c'est la vie sans gaz oxygène libre '. It was in 1861 that he suggested this view in connection with the butyric fermentation, and he returned to it in 1863 and 1876, but it met with opposition at the hand of his opponent Liebig, who in 1869 supported, with a slight modification, his own, old hypothesis. In the minds of several there was a suspicion that fermentation might come about through the agency of some soluble ferment or enzyme rather than necessarily through the vital activity of yeast. This idea had been supported by Moritz Traube (1858). In spite of many attempts, including those of Lüdersdorff (1846) and Pasteur himself, no one succeeded in demonstrating such an enzyme.

This would represent the state of knowledge in 1880, and so it remained till 1897 when Edouard Buchner produced zymase from yeast juice, and found it was capable of inducing the alcoholic fermentation in certain sugars. A long list of workers might be mentioned who confirmed Buchner's observations, which were regarded as marking the end of a discussion which had been going on for a century. The result of the modern work has been to show, however, that the problem is not so simple as it at first sight appeared, for factors other than zymase are now known to be necessary before it can induce its effect. In papers from 1904 onwards Harden and Young have shown that zymase action depends on the presence of a second factor termed ' co-enzyme ', which is

cocto-stable, and a phosphate also is essential. The modern development of the problem cannot be further referred to here, but may be consulted in the works of Harden (1923) and Delbrück (1925).

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Spontaneous Generation.

Animals not manifestly produced by the act of generation were presumed by the ancients to come into existence by the combined action of heat, air, earth and putrefaction. This doctrine received concrete shape from Aristotle, who in his ' Historia animalium ' described several instances of alleged spontaneous generation. The idea, however, existed centuries before his time in connection with the sudden appearance of swarms of bees in animal carcasses (see Judges, Chapter XI). It is

interesting to note, however, that Homer knew the real origin of larvæ from flies (Iliad, XIX, 23–27). The doctrine of spontaneous generation persisted through the Middle Ages, and, as late as 1652, J. B. van Helmont, the chemist, actually gave a recipe for the spontaneous generation of mice. It consisted in stuffing a dirty piece of linen into the mouth of a vessel containing corn. Within a matter of 21 days, according to him, the fermentation from the dirty garment disappears, and is changed by the smell of the corn into mice. What was most surprising to van Helmont was that the mice thus generated were of adult size, fully formed and capable of copulating with other mice the product of their parent's seed.

Statements such as these were rendered highly improbable by the experiments of Franceso Redi (1626–97) the poet physician of Arezzo. In his famous ' Esperienze intorno alla generazione degl' insetti ' (1668) he related many ingenious experiments which showed that maggots in meat are really the offspring of flies. He placed meat or fish in large vessels covered with the finest Naples gauze, and for further protection he placed the vessels under a gauze-covered frame. Many flies were seen moving about on the latter, but no maggots appeared in the meat itself. Sometimes he saw flies deposit their ova on the gauze and even in the air before they alighted. He also showed that when meat was allowed to become wormy, and the vessels were then closed, the maggots developed into flies of different kinds. Despite these results it is surprising that Redi believed that the insects in galls on flowers and trees are generated spontaneously, and it took the observations of Vallisnieri (1700) to demonstrate the error of this. Other observers showed that creatures like weevils and fleas do not come from corn or dirty blankets as was the belief of the time.

The idea of spontaneous generation, however, still persisted and was a theme for discussion among physicians and philosophers (Sir Thomas Browne, 1646 ; Alexander Ross, 1652), but gradually died down as far, at any rate, as concerns the more highly organized animals. There were many who still continued to hold that spontaneous generation was the rule in regard to the vast world of microscopic creatures which had been revealed by the lenses of Leeuwenhoek from 1675 onwards. Leeuwenhoek himself believed that the animalcules which appear in infusions really come from the air, where they are present in the form of seeds or germs. To show that they do not come from the matter of the infusion itself, his contemporary Louis Joblot (1718) heated infusions, and he apparently was the first to make this kind of experiment, as early as 1710. Hay infusions in cold water and exposed in open vessels soon teemed with animalcules. He then boiled hay infusion for 15 minutes and put equal quantities into two vessels. One of these he closed, before it was cool, with well-soaked parchment : the other was left open. In the latter, animalcules appeared, but none in the closed vessel. At the end of the experiment the parchment was removed and the infusion was soon peopled with animalcules.

The experiments of Joblot were not regarded as conclusive, and spontaneous generation continued to be held as a fixed belief, and indeed acquired renewed interest through the writings of John Turberville Needham and the Comte de Buffon. In his famous ' Histoire naturelle ', which began to appear in 1749, Buffon developed the idea that vitality is an indestructible quality. He considered that all living matter is composed of ' organic molecules ' or ' parts ', in themselves unalterable but capable of entering into the most diverse combinations. He regarded spontaneous generation as a consequence of his theory of organic molecules. Needham, who was an English Roman Catholic divine, came into touch with Buffon, and collaborated with him for some time. He studied in particular the microscopic appearances of germinating seeds, and observed immense numbers of moving particles of the smallest dimensions (Needham, 1749). In order to prove that these particles really come from the infusion itself and not from without, he made what he considered to be a crucial experiment. ' I took ', he says, ' a quantity of mutton gravy hot from the fire and shut it up in a phial closed with a cork so well masticated that my precautions amounted to as much as if I had sealed my phial hermetically '. He even heated the corked vessel on hot ashes, but in some days it swarmed with animalcules. He examined many different infusions but the result was always the same. Needham believed that in every microscopic particle of organic matter there resided a ' vegetative force ' which permitted the organic matter of the infusion to spring into life. The idea of a vegetative force was not a new one, but under different names had been argued in metaphysics from the time of Democritus down to Moreau de Maupertuis (1746). The statements of Buffon and Needham were ruthlessly criticized by an anonymous writer (1751), now known to have been the French metaphysician, Lelarge de Lignac, and by Voltaire (1764 ; 1768 ; 1769). The principal blow came, however, from the great Italian naturalist and experimenter, Spallanzani, who published two separate researches on the subject (1765, 1776). It is not possible in the space at our disposal to do justice to the hundreds of experiments carried out by Spallanzani. It must suffice to say that he placed infusions of many different kinds into flasks, which were then heated. He tried various methods of closing his flasks, such as cork, wood and paper, but was not sure that he had effectually excluded the air, in which he inferred that the animalcules which appear in infusions are carried. Ultimately he resorted to hermetic sealing and found that after the application of heat, the bigger the flask the quicker appeared the animalcules. He made fresh infusions and having removed the air by the air pump he boiled the infusion and distributed it into flasks which had previously been well heated. He then closed them hermetically. As a result of these long-continued experiments, the Abate Spallanzani concluded that it was not enough to seal the flasks hermetically. In addition the included air must contain no animalcules or their ' germs '. If provision is made against this contingency and everything is carried out properly animalcules

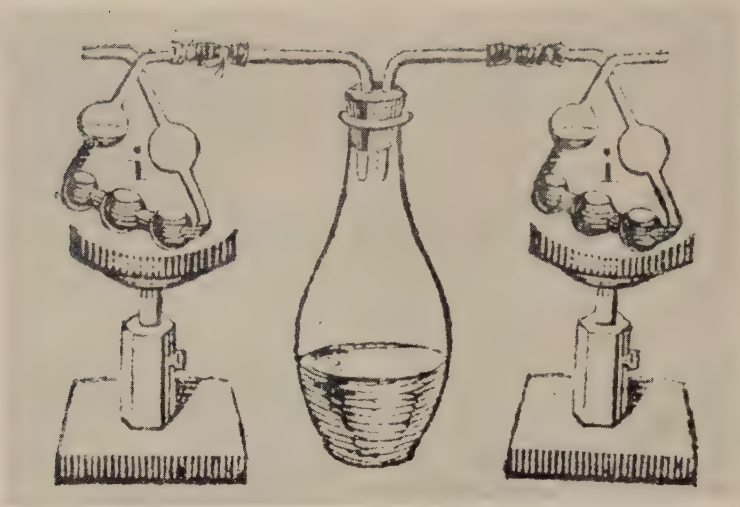

FIG. 4.—Experiment of Franz Schulze. From Schulze (1836).

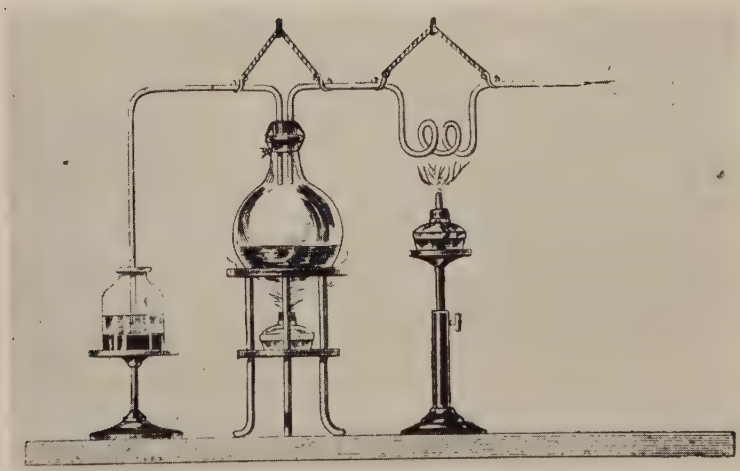

FIG. 5.—Experiment of Schwann (1837).
Fig. copied from Watson Cheyne's *Antiseptic Surgery* (1882).

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never appear in well-boiled infusions, unless air somehow or other has entered the flask and come in contact with the infusion. Spallanzani's beautiful experiments ought to have brought the discussion on spontaneous generation to an end, but Needham criticized them on the ground that the prolonged heating had enfeebled or annihilated the ' vegetative force ', and had rendered the air incapable of vivifying the organic infusion. It was to answer these criticisms that Spallanzani (1776) returned to the question, and, in a large series of experiments conceived with great ingenuity, he answered almost every objection that had been raised by Needham. Spallanzani, however, was in the position of trying to prove a negative, and thus it was that at the end of the eighteenth and well into the nineteenth century the doctrine of spontaneous generation continued to be held by most of the leading naturalists of the time.

It may be pointed out that the terms ' spontaneous ' or ' equivocal ' generation have not always been used in the same sense nor has the word ' heterogenesis ' introduced any clarity into the question. By some writers it was held that spontaneous organization of dead or inorganic matter may occur without the agency of a previous living thing. This, for example, was the belief of Bastian (1872) with his ' archebiosis '. Others such as Needham and Pouchet were professed vitalists in the sense that they considered some pre-existing vital force to be necessary for the production of new life. Pouchet, for example, never believed that animalcules can appear *de novo* in solutions of mineral ingredients. In his opinion they only appeared in organic infusions the matter of which had previously been alive, and which in some mysterious way still retained some kind of vital force.

In 1836 Franz Schulze, working in the laboratory of the chemist Mitscherlich, performed an important experiment on the relation of air to the question of spontaneous generation and putrefaction. He took a flask (Fig. 4) half filled with a vegetable infusion and furnished with a good cork perforated to receive two bent glass tubes fitted air-tight. The infusion was well boiled over a sand bath, and while steam was issuing freely from the two tubes he attached to each an absorption bulb (i) the one containing concentrated sulphuric acid and the other a solution of caustic potash. The whole apparatus was exposed on a window sill and side by side, as a control, with a similar infusion directly open to the air. From the end of May to the beginning of August, Schulze applied his mouth to the open end of the potash bulb several times daily and aspirated the air slowly out of the infusion flask. Fresh air to enter had to pass through the sulphuric acid. No infusoria appeared in the infusion although when exposed at the end of the experiment it rapidly teemed with confervæ, infusoria and moulds. The control flask even on the second day of exposure had shown growths of vibrios and monads, and later, of infusoria and rotifers. The experiment of Schulze is important as being the first one to show that air in the flask is not the cause of the growth in the infusion, and its crucial nature has been recognized by all subsequent

workers. It has been repeated many times, but often with variable results. Tyndall (1878) carefully analysed the causes of failure, and showed that success depends on the fact that the entering air must traverse the sulphuric acid very slowly so that the floating matter up to the core of each air bubble comes into contact with the surrounding liquid. Tyndall showed in fact that the sulphuric acid or the potash can be replaced by water provided that the air in the infusion flask is replenished slowly.

Important experiments were carried out on the relation of air to fermentation and putrefaction by Theodor Schwann in 1836 and 1837. His final experiment consisted in permitting only heated air to enter the flask containing the infusion. The general arrangement of his experiment is seen in Fig. 5.

Into a three-ounce flask about a quarter filled with meat and water was inserted a tight-fitting cork, well covered with rubber solution, and tied in with wire. The cork was traversed by two small tubes, the one entering a vessel of mercury covered with a layer of oil. The other tube was made to take a pair of spiral twists. The infusion was then boiled and during ebullition the spiral and adjacent parts were heated to the point of softening of the glass and this was continued during the cooling of the infusion so that only heated air could enter the latter. The orifice of the tube was then sealed. From time to time the air in the flask was renewed and heated air allowed to enter. Infusions treated in this way were found to remain barren for even six weeks. When the apparatus was opened at the end of the experiment putrefaction of the meat rapidly took place. That the application of heat to air was not detrimental was shown by causing frogs to respire air which had been previously heated. Schwann's experiment showed that what brought about putrefaction was not air as such, but something in the air. He considered that the germs of moulds or infusoria which are present in the air are destroyed when the air is heated, and he explained putrefaction as due to germs which developed at the cost of the organic materials in the infusion, and which thereby produced the change which had for so long been known as putrefaction. This view was supported, in his opinion, by the fact that substances like arsenic and corrosive sublimate, which destroy life, prevent, for this reason, the occurrence of putrefaction. The effect of heated air in protecting sugar solutions from fermentation was also demonstrated by Schwann, who was the real founder of the germ theory of fermentation and putrefaction. It was during the course of the above research that Schwann independently revealed the true nature of yeast. Helmholtz (1843), by experiments similar in principle to those of Schwann, confirmed the results of the latter. He considered, however, that putrefaction might be due not to micro-organisms themselves, but to exhalations from putrid substances in the air, and to settle the question he endeavoured to separate the microbes from the supposed exhalations by means of a barrier composed of a piece of bladder. A wide test-tube was filled with an infusion, and on the open end was tied a piece of bladder. The tube was sterilized and

then inverted into some putrid fluid in an open vessel. Putrefaction occurred in both fluids, and under the belief that the bladder would effectively bar the passage of micro-organisms, Helmholtz concluded that putrefaction could occur independently of living organisms. This experiment may be taken as the starting-point of many subsequent fallacious results with filters.

Experiments of great importance on the source of growth in organic infusions were carried out by Schröder and von Dusch (1854), and by Schröder alone (1859, 1861). Instead of heating the air or drawing it through sulphuric acid, they resorted to a new technique, viz. : filtration of the air through cotton-wool. The use of this substance had been suggested shortly before by Loewel (1853) in connection with super-saturated solutions. Loewel found that the crystallization which happens in super-saturated solutions exposed to air is prevented if the air is made

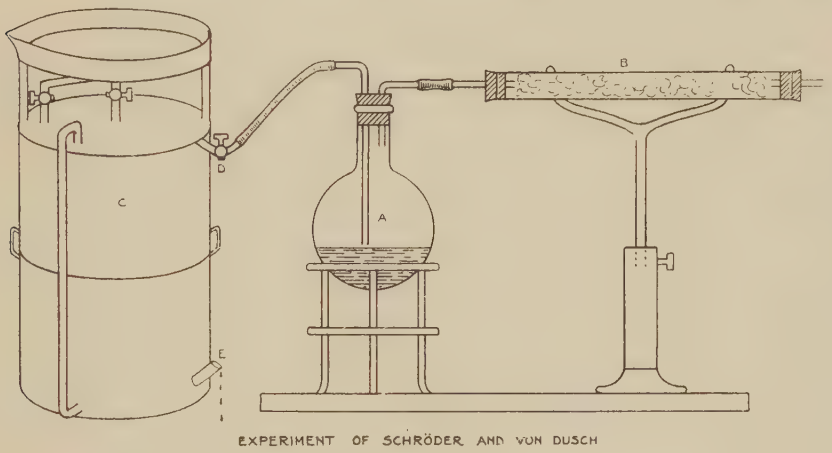

Fig. 6.—Experiment of Schröder and von Dusch (1854).
Fig. drawn for this work from author's description.

to traverse a thick layer of cotton-wool. From this it occurred to Schröder and von Dusch that a freshly boiled organic infusion might be protected from putrefaction or fermentation if the air passing into it were filtered through cotton-wool, and they constructed an apparatus to test this. (Fig. 6.)

A glass flask A containing the material to be tested was closed by a tight-fitting cork dipped in hot wax before insertion. The cork had apertures for the reception of two glass tubes bent at right angles. One of the tubes was for conducting the air into the flask and the other for leading the air away. By means of a short length of rubber tubing the conducting tube was connected with a narrow glass tube which opened into a large glass tube B (20 inches by 1 inch) through a well-fitting cork, and at the other end of the wide glass tube was another small tube open to the air. The large tube was filled with cotton-wool which had previously

been heated in a water bath. The exit or suction tube from the flask reached almost to the level of the infusion, and at its upper end was connected with the upper tube of an ordinary gasometer C provided with a stopcock D. The gasometer was filled with water and by opening the obliquely entering lower tube E and the stopcock D it acted as an aspirator. After the gas tightness of the whole system was tested the infusion was boiled vigorously. The cock of the gasometer was opened so that water ran away in drops. Meat boiled in water and placed in the apparatus described and with fresh air being continuously drawn through the cotton-wool, was preserved unchanged for 23 days. Similar infusions exposed to unfiltered air became putrid. In flasks covered directly with a mass of cotton-wool over the opening boiled meat remained unchanged. Schröder and von Dusch were, however, not successful in preventing the contamination of milk or of meat without water, even when the entering air had been filtered through cotton-wool.

Schröder (1859) continued the investigation alone and now used flasks which were plugged with cotton-wool. Egg-albumen, boiled in such flasks, remained unchanged, but yolk could not be preserved by boiling. When, however, egg yolk was first heated to 160° C. in an oil bath and was then boiled with water in a plugged flask it remained sterile. Schröder (1861) made a further study of the conditions whereby milk, yolk and meat can be preserved, and he found that if these substances were heated in sealed tubes in the oil bath at 130° C. and were then boiled in a flask with a cotton-wool plug they remained sterile. The same result was obtained by heating in a Papin's digester under a pressure of 1 to 5 atmospheres. Even very prolonged boiling at 100° C. sufficed to sterilize the refractory substances. Schröder did not believe that the germs which are associated with putrefaction originate spontaneously. They came, in his opinion, from the materials of the infusions and originally from the animal tissues themselves.

At the time that Schröder was publishing his papers, F. A. Pouchet began to present to the Academy of Sciences in Paris a series of communications in which he claimed that he had proved the existence of heterogenesis. His principal experiment consisted in taking a stoppered flask filled with water and inverted over mercury. A mixture of oxygen and nitrogen was then passed in so as to displace three fourths of the water, and some hay previously heated to 100° C. was introduced. At the end of several days there was a dense vegetation of moulds, amœbæ, vibrios and monads. Pouchet considered that their advent was spontaneous, but in this conclusion he was very adversely criticized by several of the leading members of the Academy including Claude Bernard (see Payen, 1858). Notwithstanding, Pouchet (1859) published his ' Hétérogénie ', a systematic and elaborate work running to nearly 700 pages. Pouchet was a well-known naturalist, and at the time of the publication of his work was director of the Natural History Museum in Rouen. In the preface to his book he says ' when by meditation it was evident to me that spontaneous

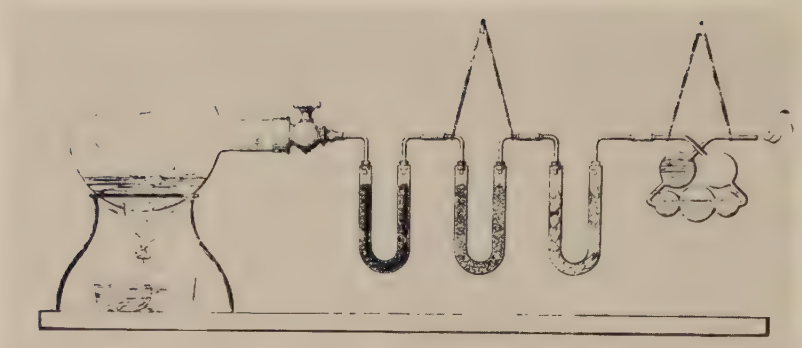

Fig. 7.—Pouchet's modification of apparatus of Schulze. Flask containing water and with small tube of hay or other material (in the neck of the flask). Three U tubes containing respectively pumice and sulphuric acid, caustic potash, cotton-wool. The Liebig bulb filled with concentrated sulphuric acid. From Pouchet (1864).

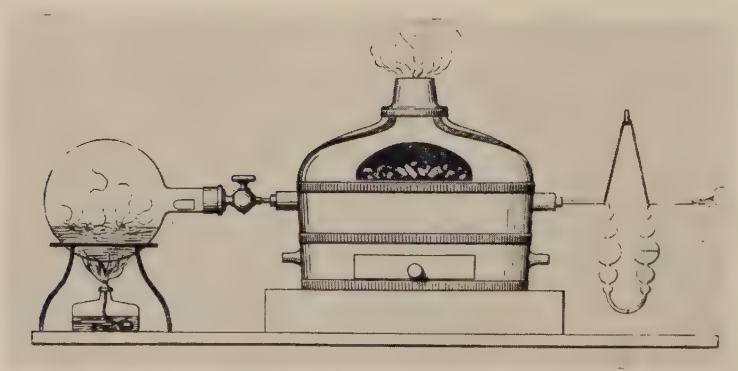

Fig. 8.—Pouchet's modification of experiment of Schwann. Arrangement of flask as in Fig. 7. Connected with the flask is a tube heated in a furnace and ending in a Liebig bulb filled with concentrated sulphuric acid. From Pouchet (1864).

To face p. 35.]

generation was one of the means employed by nature for the reproduction of living things, I applied myself to discover the methods by which it takes place '.

Starting with this idea Pouchet first tried to refute the doctrine of Bonnet (1762) and Spallanzani (1765) of panspermism. According to Pouchet the factors which favour heterogenesis are (1) organic matter in mass or solution, (2) water, (3) air and (4) a suitable temperature. Variations in any one of these were believed by him greatly to influence the type of organisms which appeared in the infusions. He brought forward experiments in which each of the supposed factors necessary were eliminated in turn, and he regarded it as proved that life may arise even although anything previously alive in the infusion in the air or in the water had been destroyed. Like previous experimenters he admitted that the real point at issue was whether germs exist in the air or not. He repeated with modified technique (Figs. 7 and 8) the experiments of Schulze and Schwann, and obtained results the reverse of theirs. In spite of all his precautions to suppress everything living in the air, he was constantly confronted with what appeared to be spontaneous generation. The infusions, heated or not, protected from the air or not, invariably teemed with living creatures, which he said were ' the offspring of death and the embodiment of life '.

The work of Pouchet aroused great interest in scientific circles, particularly in France, and among the champions who entered the lists to dispute his interpretations was Pasteur, who was already well known through his researches on fermentation. The source of ferments had not been worked out by Pasteur, and the study of heterogenesis was a matter of great importance to him. The question really resolved itself into this : are ferment germs produced immediately from the decomposition going on, or mediately from germs for which the fermentable matter is merely a suitable pabulum ? Pasteur (1860) published several abstracts of his researches as they progressed. These abstracts were expanded in his famous ' Mémoire sur les corpuscles organisés qui existent dans l'atmosphère ' (1861). This work inaugurated an era in experimental bacteriology.

Pasteur first of all attempted to demonstrate that the air really contains germs. For this purpose he fitted up a kind of aspirator (Fig. 9) in the form of a T-shaped tube R connected above with a cistern and below with an outflow tube. The aspirator being set in action was connected with a glass tube, T, passing through a hole in a shutter, F, and open to the air outside. In the glass tube was inserted a plug of gun-cotton, *a b*, which acted as a filter to arrest the aerial germs. At the end of the experiment the gun-cotton was removed and placed in a sedimentation tube, to which were added alcohol and ether, so as to dissolve the gun-cotton. The insoluble matter retained in the gun-cotton was allowed to settle at the bottom. At the end of 24 hours the deposit was found under the microscope to contain a number of small round or

oval bodies indistinguishable from the spores of minute plants and the ova of animals. The number of these bodies varied according to the temperature, moisture, movement of the air and the height above the surface of the earth. Pasteur next repeated the experiment of Schwann with heated air. For this purpose he took a flask (Fig. 10), and having bent the neck almost horizontally he placed in it distilled water containing

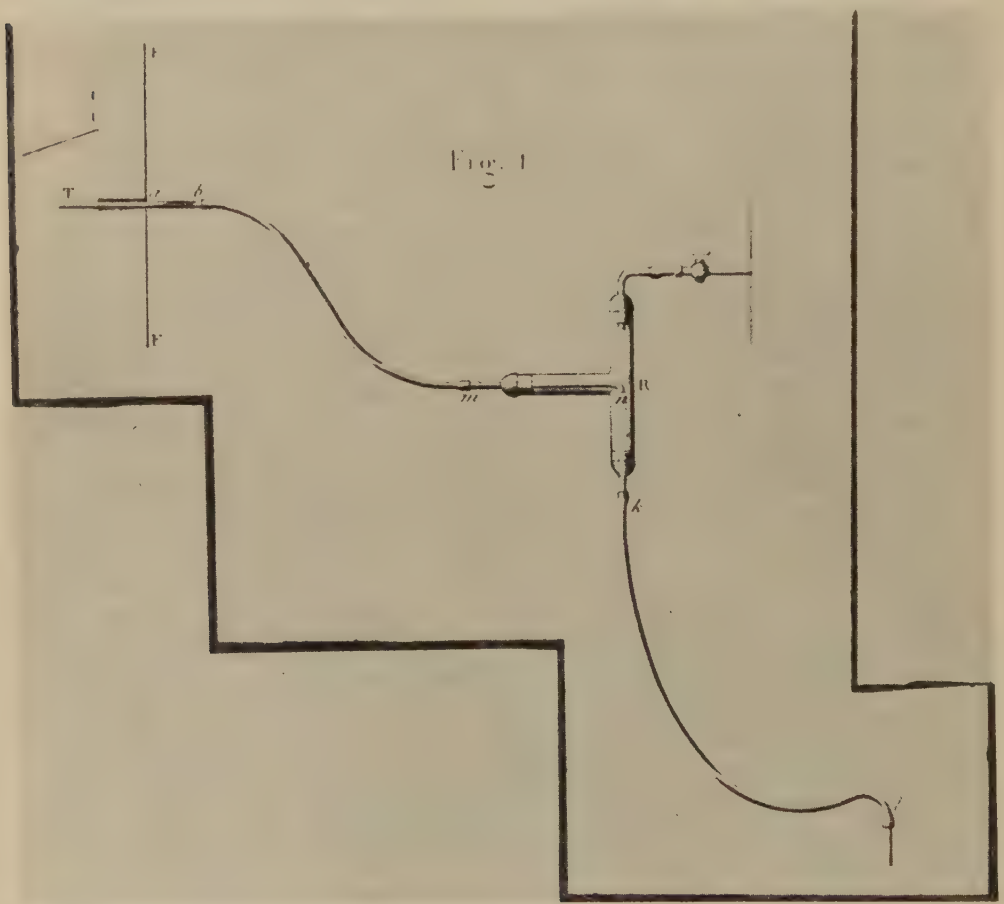

Fig. 9.—Pasteur's method of collecting dust from the air. From Pasteur (1861).

10 per cent. of sugar and 0·2 to 0·5 per cent. of the nitrogenous and mineral matter obtained from yeast. The neck of the flask was then drawn out so that it could be connected with a platinum tube capable of being maintained at red heat in a furnace. The contents of the flask were boiled for 2 or 3 minutes and allowed to cool. Air entered but it had first to traverse the red-hot tube of platinum. Finally the neck of the flask was hermetically sealed. With this technique no organisms appeared in the infusion. In previous experiments with a different technique he had not always been successful. Thus in some cases he boiled the infusion in the

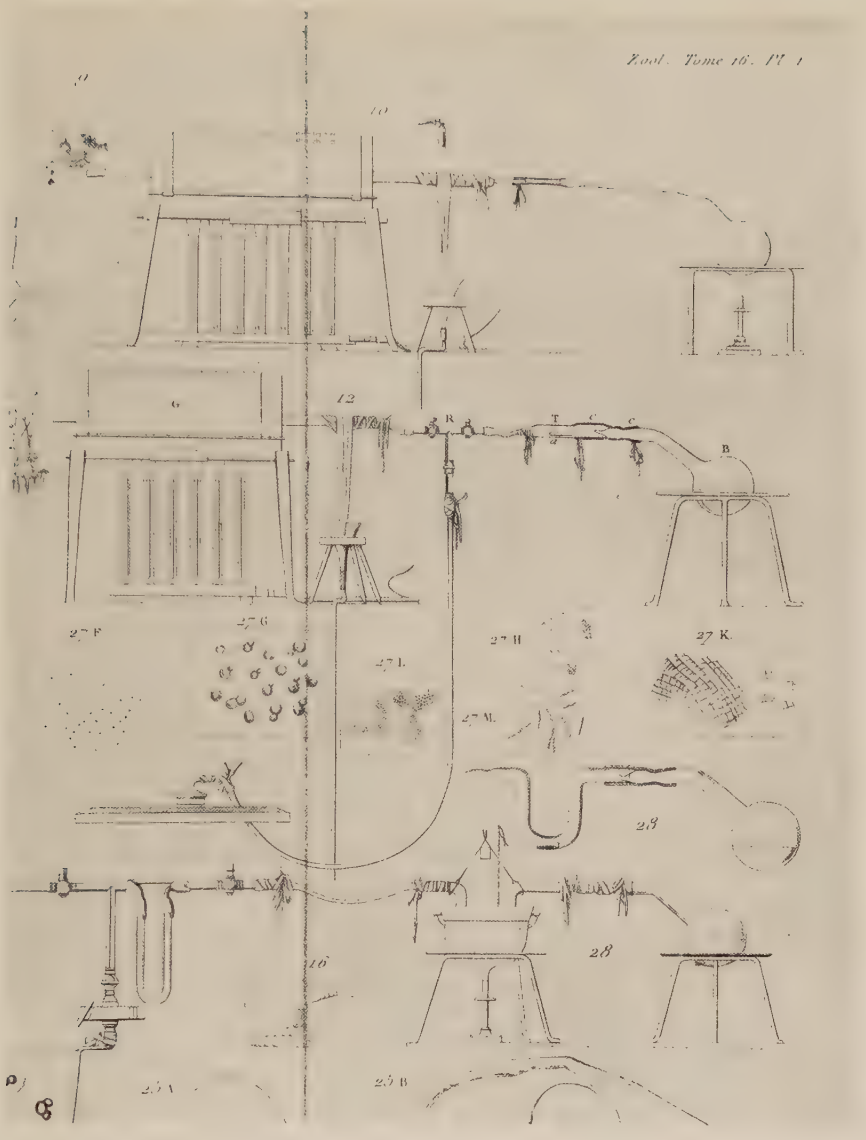

FIG. 10.—Various forms of apparatus used by Pasteur in connection with spontaneous generation studies. From Pasteur (1861).

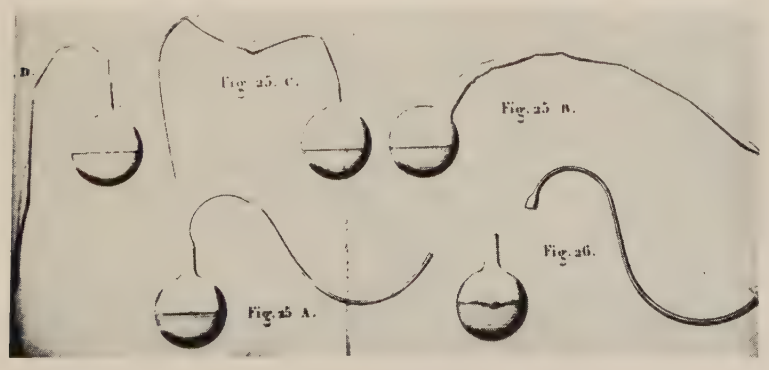

FIG. 11.—Forms of Pasteur's bent-necked flasks. In Fig. 26 the flask is represented with neck cut off and shows growth in medium as a consequence. From Pasteur (1861).

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flask, which was then sealed. Subsequently the neck of the flask was broken under mercury and oxygen which had traversed a red-hot platinum tube was allowed to enter. All the infusions prepared in this way showed growths, and he was led to infer that the source of the contamination was germ-laden dust from the surface of the mercury.

Having shown that germs or bodies like them do really exist in the air, and that it is possible to protect boiled organic infusions from contamination, if they are supplied with air which has been heated, Pasteur proceeded to show that a sterile infusion which has been boiled becomes infected if dust, the deposit from air, is introduced into it (No. 12 on Fig. 10). A sealed sterile flask, B, with infusion was connected with a glass tube containing a pledget, *a*, of gun-cotton or asbestos from an air filter. The whole apparatus was connected with a vacuum pump and a red-hot platinum tube in such a way that when the air had been evacuated by the pump only air that had passed the red-hot platinum tube was allowed to enter. When this had been done several times the neck of the flask was broken through the rubber tubing at *c* and the small glass tube, *a*, was manœuvred so that it glided into the infusion in the flask, B, which was then sealed. The constant result of experiments made in this fashion was that the infusion hitherto pure became contaminated in 36 to 48 hours. When the germ-laden asbestos was heated it could be introduced into the infusion without producing contamination of the latter (No. 28 on Fig. 10).

Pasteur also showed that boiled infusions may be kept sterile in *open* flasks, provided that the neck of the flask is drawn out and bent down in such a way that germs from the air cannot ascend (Fig. 11). This type of experiment, which had also been employed by H. Hoffmann (1860), removed all criticism that the air as such activates an organic infusion into life. If the necks of such flasks which have remained sterile for months are cut off, the infusion rapidly becomes peopled with organisms. Pasteur showed, further, that germs are not distributed with equal frequency in all samples of air. When sterile infusions were exposed to the air of the cellars of the Paris Observatory contaminations only occasionally took place. Pasteur carried out experiments of this kind at Dôle, on Mont Poupet (2,550 feet high) (Jura), and in Chamonix on Montanvert (6,276 feet) and on the Mer de Glace. At the level of Dôle 8 flasks out of 20 exposed showed growth, whereas on the Mer de Glace only 1 flask out of 20 exposed showed growth. Some of the 19 flasks that remained sterile in 1860 have been preserved and are still sterile after 70 years.

These experimental results of Pasteur were regarded as having definitely negatived the idea of heterogenesis, but Pouchet remained unconverted or made it his business to dissent from Pasteur. With Joly and Musset (1863) he clambered on the Maladetta Glacier (Pyrenees) to greater altitudes than Pasteur had attained on the Alps. At 9,000 feet the heterogenists exposed infusions with the precautions advocated by Pasteur but all of them corrupted. Through the services of an Alpine climber, Pouchet

obtained air from the summits of Mont Blanc, the Buet and Monte Rosa. He inverted the bottles containing the Alpine air in a boiling solution of clover, but growths appeared in them. Everywhere his results were opposed to those of Pasteur, and caused great animation in the Academy of Sciences in Paris, where many of the leading scientists took Pasteur's side against him. It was ultimately arranged that an experimental test should take place between the opponents. In Chevreul's laboratory in the Natural History Museum in the Jardin des Plantes, Pasteur made a convincing series of experiments before the Commission appointed to decide the matter. Pouchet declined to make experiments and only raised quibbling objections. Shortly after, he published another work on heterogenesis (1864), but this gained him no credit, and he then gave up the problem of heterogenesis altogether. Others, however, took up his cause and among them may be mentioned Hughes Bennett (1868) and especially Charlton Bastian (1872). On the side of Pasteur were William Roberts (1874), John Tyndall (1876, 1877), Ferdinand Cohn (1876) and J. Lister (1878).

Owing to the varying results obtained by heated air, sulphuric acid, gun-cotton and boiling, Hughes Bennett (1867–8) devised experiments to try the effect of all these agents at once, except the first. The general arrangement of his technique is seen in Fig. 12 and the legend will explain its purport. This experiment, apparently well conceived, had, according to present experience, flaws which at that time were deemed incredible.

It was in 1872 that the English heterogenist Bastian entered the field with his large book of more than 1,000 pages entitled ' The beginnings of life ', and with unparalleled persistence he maintained the doctrine of heterogenesis for nearly forty years, during which he published many controversial papers and six other books on the subject. It may be said that at the end he fought alone for the establishment of his cherished doctrine. It is not easy to account for Bastian's position unless we regard him as a master of dialectics—a mode of reasoning which makes fallacies pass for the truth. In some ways his life-work was of service because through his experiments it was established that sterilization by heat was often much more difficult than had been indicated by the work of Pasteur. Some of his experiments were suggestive, but there can be no doubt to-day that his technique was defective, and was shown to be so, even if Bastian himself remained unconvinced.

In researches on the air, John Tyndall, the English physicist, had been impressed with the difficulty of removing the motes that float in the atmosphere. He found that if powerful heat was applied from a large burner the motes disappeared, and no light was scattered when a beam of light was passed through the air. In Tyndall's words the air had become ' optically empty '. He thus began to employ a beam of light to reveal dust and gradually drifted into the discussion on heterogenesis. For the purpose of studying the development of organisms in organic infusions, Tyndall (1876) devised a special chamber (Fig. 13) made of wood and

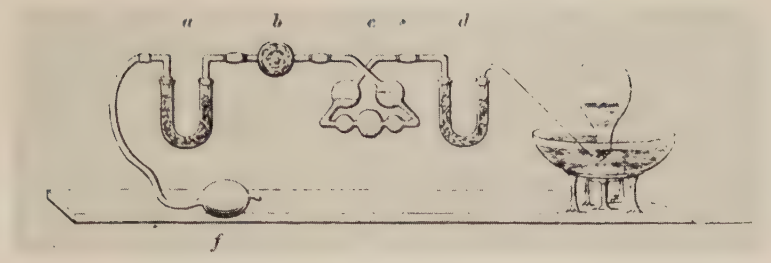

FIG. 12.—Experiment of Hughes Bennett. *a*, Bent tube containing liquor potassæ and pumice; *b*, a hollow glass ball filled with gun-cotton; *c*, Liebig's bulbs containing sulphuric acid; *d*, bent tube containing sulphuric acid and pumice; *e*, infusion kept at boiling-point when air was pumped into it from the india-rubber ball *f*. From Hughes Bennett (1868).

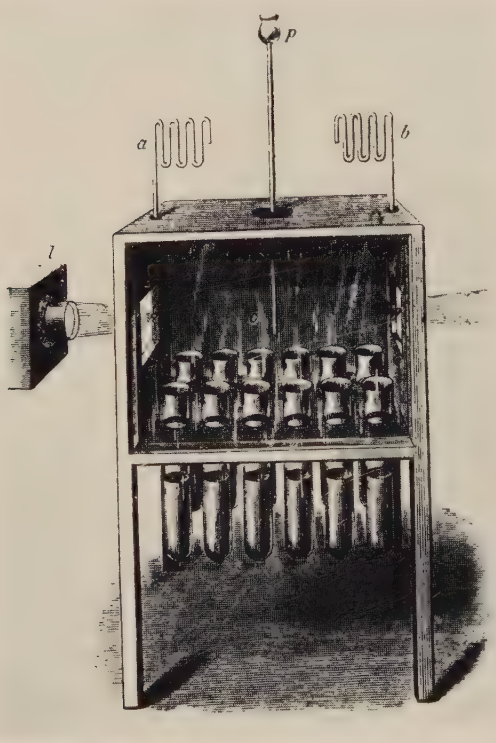

FIG. 13.—Tyndall's culture chamber. From Tyndall (1881).

[*To face p.* 38.

provided with a glass front. Two glass windows were inserted in the sides, while the back was provided with a small door. The top was perforated in the middle by a hole 2 inches in diameter and this was closed air-tight by a sheet of rubber pierced in the middle by a pin-hole through which was pushed a long thistle-ended pipette, *p*. A circular tin collar 2 inches in diameter and $1\frac{1}{2}$ inches deep surrounded the stalk of the pipette, the space between being packed with cotton-wool moistened with glycerin. Into two other small apertures, *a* and *b*, in the roof of the chambers were inserted the open ends of two narrow tubes connecting the interior of the box with the atmosphere. The tubes were bent up and down several times so as to intercept and retain particles carried in the air by changes of temperature. The floor of the chamber was pierced with holes to take (6–12) test-tubes intended to contain the nutrient fluids exposed to the moteless air. The chamber was left standing for several days until a beam of light passed through the lateral windows showed that the floating matter in the air had been deposited on the interior surface, where it was retained by a coating of glycerin with which these surfaces had been previously coated. The pipette having been manœuvred into the test-tubes the fluid to be tested was poured into the thistle end and allowed to enter the tubes until they were nearly filled. The tubes were then immersed in a boiling brine bath and kept at boiling-point for five minutes.

Tyndall used a very large number of different infusions, and as controls, similar infusions, but exposed to air. With increasing improvement in his technique the ' protected ' infusions in the chambers remained unaffected for months, and it was thereby established that the power of developing life by atmospheric air and its power of scattering light go hand in hand. Tyndall also made experiments with filtered and with calcined air in hermetically sealed glass bulbs. In 130 experiments made with bulbs heated for 3 minutes in the oil-bath 100 remained sterile. A large number of sealed bulbs with sterile infusions were opened by Tyndall in various parts of the Royal Institution, Albemarle Street, London, and for him by Charles Darwin in his study at Downe, and by J. D. Hooker at the Botanical Gardens in Kew. All exhibited growths.

In his second research (1877[1]) Tyndall studied the question further, and especially in relation to the remarkable results obtained by W. Roberts (1874) and F. Cohn (1877). Roberts had found that when hay infusions are carefully neutralized with alkali they can withstand three hours' boiling before they are rendered sterile. Pasteur (1861) had already shown that whereas acid infusions have their germinal life destroyed at 100° C., a temperature of over 100° C. was required to sterilize infusions with an alkaline reaction. Cohn (1877) confirmed these observations in part. Tyndall now investigated this question thoroughly. After a tedious inquiry involving hundreds of experiments it transpired that infusions made of old dried hay were in general much more difficult to sterilize than those made from fresh hay. Turning to other infusions with which

he had been so successful in 1876 he now found that he could no longer sterilize them in closed chambers after 5 minutes' boiling, as he had previously been able to do. Rather than accept the suggested doctrine of spontaneous generation, Tyndall laboured to make his technique more and more rigorous. For a time successes appeared to be the exception rather than the rule. Either the old substances with which he experimented in 1876 had acquired a new or increased generative force, or some new agent was making itself felt and seemed to be, as he says, omnipresent and persistent. He now transferred his activities from the Royal Institution to the laboratory in Kew Gardens, and found that infusions which in the former could not be sterilized in 200 minutes' boiling were completely sterilized in the latter in 5 minutes. Returning to the Royal Institution he began to take renewed precautions, and finally was successful in a shed which he erected on the roof of the Institution 24 feet away from the laboratory in the Institution. Infusions which required 5 *hours* for their sterilization in the laboratory were sterilized in the shed above in 5 minutes. It was impossible to believe that spontaneous generation existed in the laboratory and not in the shed 24 feet away. The difference was ultimately ascertained to be due to bundles of old dry hay which had been lying on the floor of the laboratory and had yielded clouds of highly resistant germ dust. A simple wave of a bunch of dry hay in the shed made it as infective as the air of the laboratory. Tyndall now proceeded to determine the limits of thermo-resistance of the germs which for so long had been contaminating his infusions. Boiling for $5\frac{1}{2}$ hours was not always capable of rendering infusions sterile. In order to prove that this was not due solely to germs in the air included in the glass bulbs, but that the germs actually survived this prolonged temperature in contact with the infusion itself, Tyndall devised the following technique (Fig. 14).

A represents the shape of the glass to begin with. In B the neck is shown plugged with cotton-wool *c* and hermetically sealed at *a*. The lateral tube was drawn out at *o* and *p*, the end *n* being then connected with an air-pump. After two or three emptyings and fillings the bulb was then charged with one-third of an atmosphere of thoroughly filtered air. While the pump maintained the pressure within the bulb the tube was sealed at *p*. In this state the tube was heated in the flame nearly to redness, the end *p* was then introduced into a beaker containing the hay infusion, and pressed against the bottom so as to break it. The liquid ascended and filled the bulb two-thirds full, at which stage the constriction at *o* was sealed, the final form of the bulb being seen at C. Bulbs filled in this way were then exposed to the boiling temperature for periods varying from 10 minutes to 8 hours. All the bulbs exposed for 4 hours' boiling showed growths. Sterilization was, however, complete in all the bulbs with 5 hours' ebullition with the exception of one which withstood 8 hours' boiling and at the end showed a growth.

From the diverse results of his numerous experiments, Tyndall began to realize that bacteria have phases, one being relatively thermo-labile and

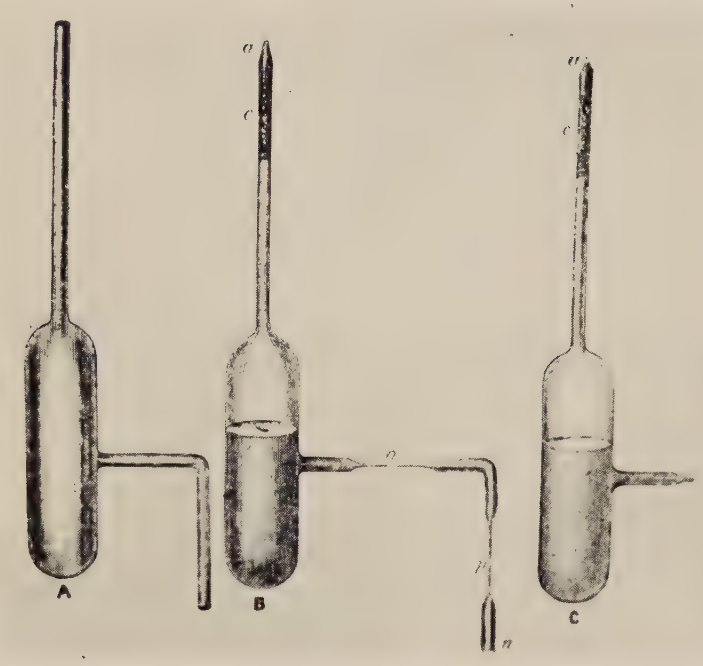

FIG. 14.—Tyndall's arrangement for demonstrating the thermo-resistance of bacteria. From Tyndall (1881).

[*To face p.* 40.

destroyed at 100° C. in 5 minutes. Another stage which he regarded as the germ of the bacterium was thermo-resistant to an almost incredible extent. This very important fact was independently discovered by Ferdinand Cohn (1876) in the same year. They arrived at the same conclusions by different methods. Tyndall carried the matter further by elaborating his method of fractional sterilization by discontinuous heating known to-day as Tyndallization. Observing that active bacteria are easily killed by boiling and that a certain period of time is necessary to enable the resistant germ to pass into that condition in which it is sensitive to heat, Tyndall (1877[2]) devised this process of sterilization and gave the first account of it in a letter (14th February, 1877) to Huxley. By it he showed that discontinuous boiling for one minute on five successive occasions could render an infusion barren whereas one single continous boiling for one hour might not.

Tyndall's researches gave the final blow to the doctrine of spontaneous generation as much as, if not more than, Pasteur's. This was probably due to the fact that his results were diffused in the form of lectures, demonstrations, papers, books and correspondence in newspapers and magazines. Whereas the personality of Pasteur inspired something of the nature of opposition, Tyndall's magnetic influence, his exact technique, the logic of his interpretations and the admirable style of his writings were acceptable to a large audience of intelligent people.

Credit must also be given to the English physician, William Roberts (1874), whose work confirmed and in some respects corrected that of Pasteur. Lister (1878) laid particular stress on the necessity of sterilizing by heat all glassware intended for experiments with organic infusions. He devised a special iron box—hot-air sterilizer—and recommended that heat should be applied at 150° C. for 2 hours.

In a research on the sterilization of hay infusions, Ferdinand Cohn (1876) showed that the growths are associated with bacillary forms of the type of *Bacillus subtilis*. He found that at a certain time the bacilli exhibit in their interior highly refractile bodies which he recognized to be spores. He showed that when this stage is reached the vegetation of the bacillus ceases in the hay infusion. The spores were, however, capable of germinating in a fresh infusion, and Cohn actually saw the act of germination whereby the spore gave exit to the young bacillus capable of progressive vegetative growth. He independently determined that the spores of *B. subtilis* are very heat-resistant, and he attributed this to their chemical composition and their physical state of dryness.

While the discussion on spontaneous generation was in progress many attempts were made to determine whether organic matters such as urine, blood and the tissues of animals and plants contain micro-organisms, and if not whether they can be preserved intact without recourse to heat or chemical disinfectants. The pioneer in this line of work was J. H. Van den Broek of Utrecht (1858–9, 1860), and following him must be mentioned Pasteur (1863, 1876), Lister (1873), W. Roberts (1874), Watson Cheyne

(1879), G. Meissner (see Rosenbach, 1880) and many others. All these workers succeeded—often after failures—in preserving a great variety of organic materials of living animals or vegetables, and many of our present beliefs were founded on these early observations. The greatest difficulty was experienced in obtaining and keeping milk in a sterile state, but this was successfully accomplished in a few instances by W. Roberts (1874), Lister (1878) and G. Meissner (Rosenbach, 1880). The few successful experiments sufficed, however, to show that milk is not inherently fermentable as had long been believed. A peculiar interest and importance attaches to the question whether living animal tissues are sterile or not. The results have not always been in accord, and many of the early workers were led astray by defective methods. Successful experiments were made by G. Meissner, the physiologist of Göttingen. His work was not published by himself, but was made known by Rosenbach (1880). After many failures Meissner succeeded in preserving unchanged whole organs of cats and rabbits. The exact technique employed by Meissner is not stated, but it is known to have involved most rigorous asepsis. Every instrument was sterilized by heat at 160° C. All water was boiled. Only sterile swabs of cotton-wool were used and the operations were carried out in a dust-proof room with the minimal number of assistants. The skin of the animal was totally reflected. In one interesting experiment Meissner preserved in a state of sterility the flesh of a kestrel which had been fed on carrion for weeks. In modern times some doubt has been thrown on the idea that the normal tissues are always sterile, and several papers have been published to indicate that bacteria may be found in organs removed from healthy animals immediately after death, and examined by a technique apparently efficient. The negative results suffice to show that animal tissues are not inherently putrescible. The result of all the work above described and carried out by so many workers sufficed to lead to the firm belief that spontaneous generation is in reality what Pasteur called it—a chimæra.

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Putrefaction.

For many centuries the idea prevailed that putrefaction is an important factor in the production of disease, and it no doubt arose from the observation of epidemics during and after wars, when large numbers of putrefying corpses were left to pollute the soil. In the early part of the nineteenth century the observation of complications in surgical wounds led to the differentiation of conditions which were known by such names as sepsis, putrid infection, and putrid intoxication, and it was generally assumed that these states were the result of the absorption into the system of putrid substances. This idea was the outcome of observations that the injection of decomposing materials determined in animals symptoms which resembled those noted in septic diseases. The earliest

experimental work on which this was founded was that of Gaspard (1822, 1824). He made some 70 experiments on dogs, sheep, foxes and pigs and injected them with putrid infusions, pus, vaccine lymph, blood, bile, semen, urine, saliva and various gaseous putrefactive products such as carbon dioxide, hydrogen, and sulphuretted hydrogen. He introduced the materials by diverse routes and minutely described the symptoms and lesions which ensued.

In the most acute cases, vomiting, excitement, convulsions and other nervous phenomena dominated the scene. Diarrhœa and heart failure were frequent. After death, Gaspard noted ecchymoses in the lungs and serous membranes. The principal lesion, however, seemed to be a congestion of the alimentary mucous membrane. Single large doses or repeated small doses of pus also caused death when injected, and this result was attributed by Gaspard to the belief that pus was a fluid in a state of commencing putridity. Among other things he observed that the blood of a dog which had died from the injection of a putrid infusion was also capable of producing a similar, if slighter, train of symptoms in a normal dog.

Gaspard's work aroused great interest, and was repeated by many others in the early years of last century. Magendie's (1823) contribution to the subject was important, in that he showed that a dose of putrid blood, lethal if administered intravenously, was inert if given by the mouth. He showed also that the toxicity of a putrid fluid was greatly diminished after filtration through paper. By placing animals on a grill above a putrid mass, he found that no ill effect resulted from the inhalation of the putrid effluvia. This observation rendered improbable the ancient and cherished belief of mankind that the penetration of evil odours into the respiratory system is a frequent cause of disease. The experimental production of sepsis and putrid intoxication continued, and P. L. Panum (1856), at that time professor in Kiel, attempted to disclose the nature of the septic poison. According to him, the characteristic picture of putrid intoxication comprises cyanosis and incontinence of urine and fæces coming on after a latent period of an hour or two. After death, the blood was found to be dark and incoagulable. Panum tested separately many known putrefactive products such as butyric acid, leucine, tryosine, ammonium valerianate and carbonate. None produced the symptoms and signs of putrid intoxication. He distilled the clear filtrates from putrid infusions and found that the distillate was inert whereas the residue was toxic. This residue was soluble in water, but not in alcohol, and it was still toxic after being boiled at 100° C. for 11 hours. From this he concluded that the septic or putrid poison could be neither a ferment, nor a living agent such as a bacterium, but must be a chemical substance. He considered that, in some way, the proteins, not in themselves active, absorbed some other agent or substance in which resided the toxicity.

Panum's observations led to a greatly increased activity in this line of research, and the publication of a voluminous literature [O. Weber

(1864, 1865); Hemmer (1866); Schweninger (1866); and others]. A very full and exact account of all the work will be found in the monographs by Gussenbauer (1882) and Hiller (1879).

The idea that putridity was really a biological process was suggested or actually demonstrated by Schwann (1837), and was confirmed by Pasteur (1863), who stated that it was due to organized 'ferments', as he called them, of the genus vibrio. The authority of Pasteur exerted a great influence on the subsequent study of the process of putrefaction. A number of workers continued to pursue enquiries intended to show that the phenomena of putrid intoxication are due to purely chemical poisons, whereas others tried to show that 'septic' conditions in the body are related to the development of micro-organisms.

Working conjointly with the chemists, Dragendorff and Schmiedeberg, Bergmann (1868) believed that the active putrid poison had been found in the substance which they called 'sepsin'. In a very large book, Arnold Hiller (1879), a strenuous opponent of the germ theory of putrefaction, contended that putridity was due to chemical ferments which broke up the organic constituents of the putrescible material into simple substances, and it was then and then only that bacteria could play any part in the process. Rosenberger (1882) supported the chemical, as opposed to the biological, view, and was able to produce putrid intoxication in animals by the injection of putrid fluids which had been heated to 140° C. for 2 hours.

In spite of these and many other researches which had been going on for half a century, the true nature of putrid intoxication seemed no nearer solution. The lethal action of putrid infusions was found to be very variable, and many organic fluids, not, strictly speaking, putrefactive, produced symptoms like those which followed the injection of substances actually putrid. This was pithily expressed by Bouley, who said, 'tout ce qui pue ne tue pas, tout ce qui tue ne pue pas'. The failure to find in putrid materials chemical substances which could reproduce the symptoms following the injection of the putrid materials themselves led to a temporary cessation of work in this direction. There was, however, a revival in the late seventies of last century with the doctrine of cadaveric alkaloids or ptomaines. The pioneer in this work was the Italian chemist, Francesco Selmi, whose first work was published in 1872. He demonstrated that many of the so-called ptomaines gave reactions similar to those of certain vegetable alkaloids. An early and persistent worker along the same lines was Armand Gautier (1881), who introduced the somewhat indefinite term 'leucomaines' for basic substances found in the living tissues (λεύκωμα, anything whitened). The principal exponent of the ptomaine doctrine was, however, L. Brieger (1881–6) who isolated and determined the composition of a number of substances of cadaveric origin. The relation of these substances to disease was for a time believed to be important but has suffered a diminishing interest.

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Pyæmia and Septicæmia.

The history of the modern ideas of what is now called 'sepsis', and which used to be called by such names as 'putrid intoxication', 'septicæmia', 'ichorræmia', 'sapræmia' and 'surgical fever', is a somewhat complicated subject which touches the general doctrines of surgery and medicine. In the previous section some indication was given of the ideas which were gradually formed to explain the effects following the injection of putrid fluids into the animal body. The clinical picture was referred to under such names as 'putrid intoxication' or 'septicoémie' (Piorry, 1837). Symptoms apparently similar to those produced experimentally were frequently observed in man, and were often associated with abscess formation in the viscera or suppurative processes in the periphery of the body. The natural supposition was that there had been an escape of pus into the blood system, and it was for this reason that Piorry proposed to call the condition 'pyoémie', anglicized later to pyæmia. Early in the nineteenth century the microscopic characters of pus were extensively studied by Donné (1836), Bonnet (1837), and many others (see Bérard, 1842). In addition, many experiments were made to determine whether the injection of pus, fresh or putrid, can produce pyæmia in animals. The works of Boyer (1834), De Castelnau and Ducrest (1846) and Sédillot (1849) may be taken as examples of this kind of investigation. In general it was not found possible to produce pyæmic symptoms with multiple abscesses, although here and there positive results were reported. The question whether pyæmia and septicæmia are one and the same or different states emerges in the article of Bérard (1842). Sédillot (1849) was fully persuaded that pyæmia is always produced by the admission of pus globules into the blood, and he supported his contention in a series of experiments and numerous clinical records.

Through the experiments of Coze and Feltz of Strasburg, new developments took place which indicated that ' infusoria ' occur in infective processes. Their papers appeared between 1866 and 1869, and were collected with additions in book form (1873). They injected various putrid substances into dogs and rabbits, and, among other observations, showed that the blood of animals thus injected produces symptoms in normal animals. They also showed that such blood increases in virulence as it passes from the body of one animal to that of another. This observation was confirmed and made widely known by Davaine (1872). In the blood, both during life and after death, after the injection of putrid infusions, Coze and Feltz found bacteria which were motile (' *B. punctum* ') or in chains (' *B. catenula* '), and they also reported the occurrence of bacteria in the blood of persons suffering from typhoid fever, variola, measles, scarlet fever and puerperal fever. They attempted to cultivate these bacteria on a medium of sugar-water containing ammonium lactate and sodium bicarbonate, but without success. The prolonged researches of Coze and Feltz, incomplete as they were, had the merit of directing attention to the bacterial, or as it was then called ' zymotic ' nature of septic conditions, but the ' Zeitgeist ' was not propitious, and thus it was that in the medical world discussions continued along the old lines. As late as 1867 Roser discussed five theories of pyæmia, which he characterized as (1) the mechanical, (2) the septic, (3) the zymotic, (4) the eclectic, and (5) the sceptical. According to the ' sceptics ' there was no such entity as pyæmia. That which had been called pyæmia was in their opinion a collection of morbid conditions, the result of phlebitis, thrombosis, leucocytosis or zymotic poisons. The exhaustive and exhausting discussion on purulent infection in the Academie de médecine in Paris (1869) will also bear testimony to the mental chaos prevailing among the French medical and surgical leaders of that time.

The so-called ' germ theory of disease ' began, however, to come into evidence and was supported by Pasteur's work on fermentation. Joseph Lister grasped for surgery the full significance of Pasteur's researches, and gradually built up his antiseptic system on the basis of the germ theory. The success of Lister's treatment of wounds did much to make the germ theory acceptable to the medical profession, and it gradually became a cherished belief. Fresh interest in the question of ' sepsis ' was also aroused by the experimental work of C. J. Davaine (1872, 1879), on what he called ' septicæmia '. Having injected a rabbit with 10 drops of putrid blood he found that death ensued in 40 hours. Starting with the blood of this animal he carried the disease through a series of 25 rabbits, and found that the virulence progressively increased to an enormous extent. The fifth animal of the series succumbed to a dose of one forty-thousandth of a drop of blood from the fourth animal, and in the twentieth generation the lethal dose of blood was one hundred millionth of a drop. In the twenty-fifth rabbit of the series the fatal dose of blood was actually one billionth (French, trillionth) of a drop. In another

series of animals, Davaine showed that the increase of virulence by the process of ' passage ' was very rapid and could mount from a fatal dose of one-hundredth of a drop in the first rabbit to one ten-millionth of a drop in the second and one-billionth of a drop in the third rabbit. Davaine made a great many other observations on this experimental ' septicæmia ', and showed that the virulence of the putrid blood bore no relation to the degree of putridity, except that as the putridity increased, the virulent element appeared to diminish. He considered that the virulent factor was identical with the ' ferment of putrefaction ', and he regarded the septicæmia produced as a putrefaction running its course in the blood of the living animal. It is true, he said, that the septicæmia is not accompanied by the characteristic odour of putrefaction, but this, in his opinion, was due to the fact that the odorous principles had been eliminated whereas the ' ferment ' remained and accumulated in the animal body, which it finally destroyed.

In some quarters Davaine's experimental results and conclusions were adversely criticized, but they were confirmed by Vulpian (1872), Clementi and Thin (1873), U. Dreyer (1874), and Feltz (1874). Vulpian also expressed the view that Davaine's septicæmia was characterized by the presence of bacteria in the blood and was nothing more or less than a parasitic disease which might appropriately be called ' bacteriémie '. The relation of bacteria to septicæmia still continued, however, to be a favourite theme of discussion among clinicians and a full account of the various views will be found in the elaborate article of Chauvel (1880–81). The subject was also reported upon by a Committee of the Pathological Society of London (1879).

By degrees the word ' sepsis ' began to lose its etymological significance as something pertaining to putridity or rottenness, and came to be used merely in the sense of infection. This change of meaning was mainly the result of Davaine's experiments referred to above, and ' sepsis ' in the sense of infection also bulks largely in the surgical writings of Lister. The connection of wounds with the general symptoms of sepsis was an old observation but was not believed to be constant. Wunderlich (1857) considered that there was a form of ' spontaneous pyæmia ', implying that there was no wound to account for it. Leube (1878), however, showed that in many such cases the local lesion was present, although not immediately apparent, and from him emanated the idea of ' cryptogenetic sepsis '. From the ordinary forms of sepsis, Matthews Duncan (1880) differentiated a special type of putrid intoxication, which he called ' sapræmia ', but this has scarcely survived except as a clinical concept.

A great impulse was given to the germ theory of sepsis by the observations of E. Klebs (1872) on the pathology of gunshot wounds. During the Franco-German war he was pathologist to the Karlsbad military hospitals and in a few months made 115 autopsies, of which 75 per cent. were shown to be cases of pyæmia or septicæmia. He carried out microscopic observations and found bacteria in almost every instance. Under the

influence of the erroneous doctrines prevailing at the time he regarded the different microbic forms as belonging to one organism which he called *Microsporon septicum*.

The final settlement of the relationship of micro-organisms to sepsis was really made by Robert Koch (1878) in his famous work on the 'ætiology of traumatic infective diseases'. This small book of eighty pages, written while its author was still in practice at Wollstein, and remote from academic influences, was quite unlike anything which had preceded it on the same subject. His object was to determine whether infective diseases of wounds are of parasitic origin or not. From force of circumstances he had to confine himself exclusively to animal experiments, but he was able to show in a manner practically conclusive that a series of diseases differing clinically, anatomically and ætiologically, can be produced by the injection of putrid materials into animals. He admitted that the work of previous investigators had rendered the parasitic nature of disease probable. Conclusive evidence had, however, not been obtained, and, in Koch's opinion, could only be obtained 'by finding the parasitic micro-organisms in all cases of the disease in question; when we can, further, demonstrate their presence in such numbers and distribution that all the symptoms of the disease may thus find their explanation, and finally when we have established for every individual traumatic infective disease the existence of a micro-organism with well-defined morphological characters'.

For the solution of these problems Koch used methods that were new or at any rate had not been employed in such work before. These included staining by aniline dyes (methyl violet, fuchsine, aniline brown) and the illuminating apparatus, invented by Abbé and constructed by C. Zeiss, in conjunction with high-class oil immersion lenses. The application of these methods by Koch had an enormous influence on all the subsequent researches on infective diseases. Koch described in great detail six different infective diseases which he induced by injection of putrid fluids into animals. These diseases he called (1) septicæmia in mice, (2) progressive destruction of tissue (gangrene) of mice, (3) spreading abscesses in rabbits, (4) pyæmia in rabbits, (5) septicæmia in rabbits, and (6) erysipelas in rabbits. He showed that these diseases vary in symptoms and pathology. The bacteria were different in form and in distribution. The susceptibility of animals was variable. Taking advantage of the latter fact he was able, in at least one instance, to separate two micro-organisms occurring together and otherwise inseparable. He was not fortunate enough to meet with the particular septicæmia described by Davaine, and he was not convinced by the theory of exaltation of virus by passage through the animal body.

Although Koch's original intention was to determine whether the infective diseases of wounds in man are of parasitic origin or not, he was unable to get material for this specific purpose. This hiatus was filled up by Alexander Ogston (1880, 1881, 1883), at that time Surgeon to the

Aberdeen Royal Infirmary. Following Koch's technique exactly he examined for bacteria the contents of 100 abscesses, acute and chronic. In the latter he found no organisms, whereas in all the former micrococci were found in groups like fish roe or in chains. He showed that the difference in the appearance of the two groups corresponded to a difference in their mode of division. Injection of pus from ' cold ' abscesses, in which no bacteria could be found, was without effect on mice, whereas pus containing cocci yielded definite lesions, in which it was shown that there was a marked increase in the number of the cocci injected. The experimental suppurative lesion was capable of being transmitted to mice in series. Pus, heated or treated with phenol, was found to have been robbed of its pathogenic powers. Ogston also examined discharges from a large number of suppurative lesions, and in all he found cocci. He made many attempts to cultivate the cocci in glass cells containing a variety of nutrient media, such as Cohn's fluid, ascitic and ovarian fluids, aqueous humor, human blood, under aerobic and anaerobic conditions. Ultimately he obtained growths by implanting pus in fresh eggs, and with the growths he succeeded in producing abscesses in mice.

From these observations he concluded that micrococci produce inflammation and suppuration. He pointed out that the cocci proliferate locally and may pass into the blood-stream. In abscesses the cocci were finally excluded by the formation of a deliminating wall of granulation tissue which arrested their further invasion, and led to their final extrusion among the pus corpuscles. Ogston (1882) gave a general statement of his views on ' micrococcus poisoning '. He differentiated two kinds of cocci : the one arranged in chains and corresponding to Billroth's *Streptococcus ;* the other, which Ogston named ' *Staphylococcus* ', was arranged in masses. He opposed the idea that pyæmia and septicæmia were blood diseases, and contended that the blood was merely the vehicle which disseminated in the body that which, without its aid, would have been merely a local process. In Ogston's opinion the variations in the course and termination of infective processes were to be ascribed to specific differences in the micro-organisms, to their virulence, to the organ or structure involved and to the susceptibility of the individual affected.

Ogston's publications, advanced and original as they were, soon exerted an influence on the surgical, pathological and bacteriological ideas on suppuration and inflammation. The main criticism brought against his teaching was that micrococci are not the only pyogenic micro-organisms. By means of gelatin cultures J. Rosenbach (1884) and Passet (1885) split up Ogston's staphylococcus into three species and also added one or two other organisms which have scarcely found a permanent place in bacteriological literature. The pyogenic properties of some micro-organisms other than cocci have, however, been definitely established since Ogston's time.

For a brief period there was active discussion whether suppuration is necessarily micro-organismal in cause. A large number of workers claimed

that chemical substances such as turpentine and croton oil may produce sterile suppuration, but they were opposed by others. A full account of this discussion will be found in the works of Steinhaus (1889) and Janowski (1889).

While it is now admitted that suppuration can be produced by various acrid or even bland substances, these substances scarcely play a part in the ordinary suppurative processes of man and animals, and it is also essentially true, as Ogston first showed, that acute suppurations and abscess formations are mainly coccal in their ætiology. Ogston's views on the relation of pyæmia and septicæmia to the local phlogogenic process have also turned out to be the correct teaching. We thus come to the end of a period in surgery and pathology which, long barren in its results, was ultimately clarified by the technical methods introduced by Koch and pursued with diligence by many of his successors.

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Specific Element in Infective Disease.

On pp. 17–22 the development of the doctrine of animate contagion was traced down to the end of the eighteenth century. In spite of the observations of the microscopists that there was a great world of animalcules, there was no suggestion that the latter might be important factors in the production of disease. Animate contagion was a theoretical rather than a practical concept. The ideas on the causation of infection and contagion had not substantially advanced since the time of Fracastoro. Indeed they were often more nebulous than those which he had expressed. The idea of specificity of disease was non-existent. It is true that in the seventeenth century Sydenham had realized that there were different diseases with proper and peculiar symptoms, and he believed that there were species of disease, just as there were species of plants. He admitted, however, that it was ' very hard to reduce all the species of epidemics into classes according to the variety of their phenomena, to decipher the idiopathic characters of each, and to accommodate a method of healing particular to each '. It is apparent, however, that he had no conception of specific causes of disease. The nosology of the eighteenth century degenerated in the hands of Boissier de Sauvages (1731, 1768), Linné (1763) and W. Cullen (1769) into mere catalogues of genera and species on a purely symptomatic basis.

A new advance began in France as a result of the deep study of pathological anatomy by P. Pinel (1802) and Bichat (1802), and culminated in the work of G. L. Bayle (1774–1816) and Laennec (1787–1826) on tuberculosis. Diseases now began to be classed on an anatomical rather than on a semeiological basis. This idea was, however, violently opposed by F. J. Broussais (1816, 1822), who attempted to set up in its place the doctrine of ' médecine physiologique '. He believed that the differentiation of morbid forms was entirely factitious. Tubercle, for example, in his judgment was merely an inflammation, and all inflammations or phlegmasiæ were due to a diminished or increased degree of ' irritation '. He fanatically opposed everything that savoured of the idea of specificity, and in virtue of the prominent position which he occupied, and the powerful character of his writings, he exerted a great influence on the medical thought of his time. His doctrines were, however, not destined to survive, and indeed even during his lifetime were completely overthrown by the great clinician of Tours, P. F. Bretonneau (1771–1862), who was the real founder of the doctrine of specificity of disease as we understand it to-day. He developed the idea that it is their nature rather than their intensity which explains the differences between morbid processes, and he showed this almost to the point of proof in the case of diphtheria and typhoid fever. During his lifetime Bretonneau wrote but little on his views, which, however, were widely disseminated by his pupil Trousseau. Indeed, Bretonneau's chief work on specificity only saw the light in 1922, when it was published and edited by a descendant, Dubreuil-Chambardel.

This treatise, begun by Bretonneau in 1828, is entirely modern in its outlook and expression, and shows that he had grasped the problem of specificity with wonderful precision. His conception of specificity was ætiological, and he made the remarkable statement that ' a multitude of inflammations are due to material, extrinsic causes, by veritable beings coming from without or at least alien to the normal state of the economy '. Strange to say he made no definite attempt to connect his ' productive agent ' of specific disease with microscopic entities or ' infusoria ' which were already well known and much discussed.

Apart from theoretical fancies like those of Benj. Marten (1720), Plenciz (1762), Acerbi (1822), A. Neale (1831), and Henry Holland (1839), few, if any, seemed to have grasped the idea that animate contagia might be related to ' animalcules '. The actual demonstration of such a possibility was reserved for Agostino Bassi (1835) of Lodi. He can justly be claimed as the real founder of the doctrine of pathogenic micro-organisms of vegetable origin. Bassi was not a trained scientist nor even a doctor, except of law. He was, moreover, pursued by poverty, failure of vision and miserable ill health. It was in these conditions, however, that he carried out his classical observations on that disease of silkworms called by the French ' muscardine ' and by the Italians ' mal del segno ' or ' calcino '. After many years of study, and in opposition to the beliefs of his time, he showed that ' calcino ' is transmissible and that it is caused by a cryptogamic fungus of parasitic character. This fungus was identified as *Botrytis paradoxa* by the Milanese botanist G. Balsamo-Crivelli (1835, 1838), who subsequently named it in honour of its discoverer, *Botrytis bassiana*. Bassi took affected silkworms, passed them through a flame, and from the sub-epidermic tissue he took a small portion on a heated pin, and successfully transmitted the disease to healthy worms. The significance of Bassi's discovery was at once realized by Balsamo-Crivelli, who anticipated that great developments would accrue from it. In general Bassi's work was confirmed by Audouin (1836), Montagne (1836), and others. Important additions were also made by C. Vittadini (1852), who successfully cultivated the fungus on honey, silkworm blood, isinglass and other substances. From failing vision Bassi was forced to give up his microscopic studies, but he continued to develop his theory of contagion in such diseases as syphilis, variola, typhus, plague and cholera, and he wrote on germicides such as heat and chemicals. It is impossible to read Bassi's works without arriving at the conclusion that he was a man of great originality, and the forerunner of Schwann, Pasteur, Koch and Lister.

Another work which exerted an influence on the development of bacteriology was ' Recherches sur la nature des mucus . . . ' by the French microscopist Alfred Donné (1837). He studied certain micro-organisms in pathological discharges particularly from the genital organs. In chancres of the glans he found animalcules ' having the form described by O. F. Müller as vibrio lineola '. Donné gave a good figure, from which it

is evident that he had before him a spirochete (probably *S. refringens*). He found it in other syphilitic lesions, but not in non-syphilitic cases, nor in normal smegma. He went further and endeavoured to find out by inoculations whether the micro-organisms were the cause of syphilis, but his results were equivocal. It was about this time (1836–7) that Cagniard-Latour and Schwann first revealed the true nature of yeast and showed its relation to vinous fermentation. Their work, together with that of Bassi and Donné, led to a further search for parasitic organisms in disease, and a stimulus was received from the remarkable ' Pathologische Untersuchungen ' published by Jacob Henle (1840), subsequently famous as an anatomist. The interest in epidemic disease was at the time quickened by the advent of Asiatic cholera on European soil in 1832 during the second pandemic of the disease. Henle's book was theoretical rather than practical, and in his own words contained ' few facts and many reflections '. The chief object he had in view was to establish the hypothesis that ' the material of contagion is not only organic but living, endowed with individual life and standing to the diseased body in the relation of a parasitic organism '. He considered three types of epidemic diseases, viz. : (1) miasmatic, such as ague ; (2) miasmatic contagious, such as the exanthemata, plague and cholera ; and (3) contagious, such as syphilis, itch and rabies. In Henle's view a miasm was a quiddity, unrecognizable by the senses, but in some way related to, or identical with, other deleterious potencies like contagia. He regarded contagia as a kind of miasm in the second generation—a miasm which had passed through its first development in the second body.

In the miasmatic-contagious diseases the contagion was eliminated from the body and conveyed to the healthy by the atmosphere (volatile contagion), or by contact (fixed contagion). The discharges from the body contain the *materies* of the infection. Henle realized that variolous pus, is pus, plus the contagion of variola, and he argued that contagia must be living. The disease breaks out only after a *stadium latentis*, in which presumably the living contagion is increasing in quantity. A small trace of variolous matter will ultimately render the whole body infective. Inasmuch as there are different miasmatic-contagious diseases Henle went on to develop the idea that there must be specific differences among contagia, and he cited in this connection the observations of Bassi on muscardine and of Cagniard-Latour on yeast. Henle realized that it would be difficult to show that his views were correct. To prove that any particular agent was the cause of an infective disease he maintained that it must constantly be found in the disease ; the constant agent must be isolated and tested in the isolated state to see whether it is capable of producing the disease. This constant presence, isolation, and testing of the isolated object or agent is the unassailable basis on which all the subsequent discoveries of specific pathogenic micro-organisms have been built. Henle's statement of the proposition contains all the elements habitually but incorrectly referred to as the ' postulates of Koch ' (1878,

1884), and it is greatly to be regretted that for many years investigators were not guided by Henle's clear conclusions, which had to be restated by Koch and his co-workers before they were finally accepted as a firm belief. It was about the middle of the nineteenth century that the terms infection and contagion became interchangeable. In former times they were considered to be different, although few were in agreement as to what constituted the difference. In general ' infection ' was applied to communication of disease from sick to healthy by a morbid ' miasm ' or exhalation, whereas ' contagion ' was used for transmission by mediate or immediate contact. In our day the word ' infection ' has largely superseded ' contagion '.

The practical work of Bassi (1835), Cagniard-Latour (1837) and Schwann (1837), and the speculations of Henle (1840), may be looked on as the stimulus which led to the microscopic examination of a great many morbid products. Within a year or two the existence of many parasitic cryptogams was recorded in diseases of the skin and mucous membranes of man and animals. Thus Schönlein (1839) discovered the fungus

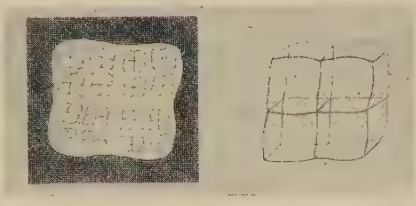

Fig. 15.—*Sarcina ventriculi*. From Goodsir (1842).

known as *Achorion Schönleinii* in certain ' Impetigines ' or favus, and Remak (1840) named it and showed its contagiosity. Buehlmann (1840) rediscovered the fungus, *Leptothrix buccalis*, previously made known by Leeuwenhoek. *Oidium albicans* was independently found by Berg (1841) and Gruby (1842). The remarkable microscopic organism called *Sarcina ventriculi* was discovered in Edinburgh by John Goodsir (1842), who published a very full account of it with figures (Fig. 15). In the same year Müller and Retzius (1842) discovered a mould of the genus *Mucor* in the lungs and air cavities of an owl (*Stryx noctua*) from Lapland. Other discoveries of great practical importance in medicine were made by David Gruby, a Hungarian, long resident in Paris. In 1843 he gave an accurate account of the fungus *Microsporum* (*Microsporon*) *Audouini* in Willan's ' porrigo decalvans ' (form of ringworm), and in 1844 he followed it with the discovery of another fungus in the ' Teigne tondante ' of Mahen. To this fungus Malmsten (1845) gave the name *Trichophyton tonsurans*.

In 1844 Mayer found a mould (*Mucor*) in the aural passage of a child aged eight, and Hughes Bennett (1844) found a similar fungus in the sputum and lungs of a case of pneumothorax. The fungus, *Microsporon*

furfur, of pityriasis versicolor was discovered by Eichstedt (1846) and Sluyter (1847). A very full account of these early observations and others will be found in Robin's ' Histoire naturelle des végétaux parasites ' (1853).

The application of the ' fungus ' theory to diseases other than the above mentioned was not so successful. In 1846 began the third pandemic of Asiatic cholera, and Europe was rapidly and extensively invaded. The time was ripe for theories and practical studies. On the theoretical side the disease was, by some, attributed to a change in the ponderable or imponderable elements of the air. Others regarded it as the result of a vegetable miasm arising from the soil. A more unlikely theory attributed cholera to volcanic or other changes in the crust of the earth. Some held it was contagious, others that it came from animalcules existent in the air. On the practical side microscopic examinations were made especially on cholera evacuations. Brittan (1849), Swayne (1849), and W. Budd (1849), all of Bristol, recorded and figured certain ' annular bodies ' which were interpreted as fungi. They were believed to be peculiar to cholera and were said to have been found by Budd in drinking-water from districts attacked by cholera. Baly and Gull (1849), who were appointed by the Cholera Committee of the Royal College of Physicians to report on the ' annular bodies ', were unable to find them in air or water. They regarded them as divers in origin and nature, and as derived in part at any rate from substances taken in food or medicine. Lastly, they found the bodies in conditions which had nothing to do with cholera, and they denied their fungoid nature altogether. In France, Pouchet (1849) found immense numbers of very ' minute animalcules ' in the rice-water stools of Asiatic cholera cases. He identified them as the *Vibrio rugula* of Müller, but made no statement on their relation to the disease.

Although he did not deal with the ultimate cause of cholera this would seem to be a suitable place to refer to the observations of the English anæsthetist, John Snow (1813–58), who in 1849 revealed for the first time the water-borne nature of the disease in its epidemic form. He was led to this view from the belief that Asiatic cholera begins in the alimentary canal, and he considered that water might be the vehicle of communication. He collected exact data of a large number of outbreaks and correlated them with water supplies. His hypothesis was not at first received with enthusiasm, but it gained ground after his exposition of the terrific epidemic of cholera which began on August 31, 1854, in Broad Street, Golden Square, London, and in the adjacent streets. In a space of 250 yards from the spot where the disease started there were 500 deaths from cholera in 10 days, at the end of which time the place was deserted by the flight of the survivors. With unerring exactitude, Snow traced the outbreak to contamination of the water of a particular pump in Broad Street. In this water he found evidences of contamination with organic matter. His views, as it happens, have turned out to be correct.

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Classification of Bacteria: Discovery of Anthrax Bacillus: Fungoid Theory, Hallier: Researches of Cohn: Billroth, Zopf: Later Systems of Classification.

The early microscopists were content to describe, often in considerable detail, the individual 'animalcules' of organic infusions, but made no attempt to arrange them on the lines laid down by Linné in the first half of the eighteenth century. The first attempt to classify 'infusoria' was made by the Dane, Otto Frederick Müller. In his 'Vermium terrestrium et fluviatilium . . .' (1773–4) he described the genera *Monas* and *Vibrio* and gave the first account of an organism, *Monas termo* (in Danish 'Grendse-monaden') which later occupied a prominent place in bacteriological literature. Müller's *magnum opus* was, however, 'Animalcula infusoria et marina . . .' (1786), published posthumously. In this magnificent work he attempted an enlarged classification. *Monas* now included ten species and *Vibrio* no fewer than thirty-one, among which one may recognize such names as *V. rugula*, *V. bacillus*, *V. undula* and *V. spirillum*.

Müller's classification was altered to some extent and expanded by C. G. Ehrenberg (1795–1876) in 'Die Infusionsthierchen als vollkommene Organismen' (1838), a large folio of 548 pages with 64 sumptuous plates all drawn by Ehrenberg himself. He included many widely separated forms in his 'Infusoria', and regarded them as possessing stomachs. They constituted his 'Polygastrica' and he regarded them as perfect and complete—'vollkommene'—as in the title of his great work. Of his 22 families of Polygastrica, the first, Monadina, the second, Cryptomonadina, and the fourth, Vibrionia, alone possess bacteriological interest and the figures he gives are reproduced in Fig. 16.

Dujardin (1841) gave a simpler classification by placing what would now be called Bacteria in one family 'Vibrioniens' with three genera, *Bacterium*, *Vibrio* and *Spirillum*. His descriptions were good, but his figures poor. M. Perty's classification (1852) was unsatisfactory and incomplete.

It was about this time that Ferdinand Cohn began to contribute those observations which did so much to establish bacteriology as a science. Cohn (1828–98) was Professor of Botany in Breslau, and a man of great talent and industry. In 1854 he published a memoir 'On the development of microscopic algæ and fungi', and suggested therein that Ehrenberg's Vibrionia family should be placed in the vegetable rather than the animal kingdom, as the members exhibited a close analogy to well-known microscopic algæ. From the lack of chlorophyll and their occurrence in infusions Cohn proposed to call the group 'Mycophyceæ' or 'Wasserpilze'. In the same paper Cohn established a new genus, *Zooglœa* (ζωον animal, γλοῖος jelly).

Cohn's opinion carried weight from the start, and, continuing his researches for two more decades, he reached the front rank as one of the

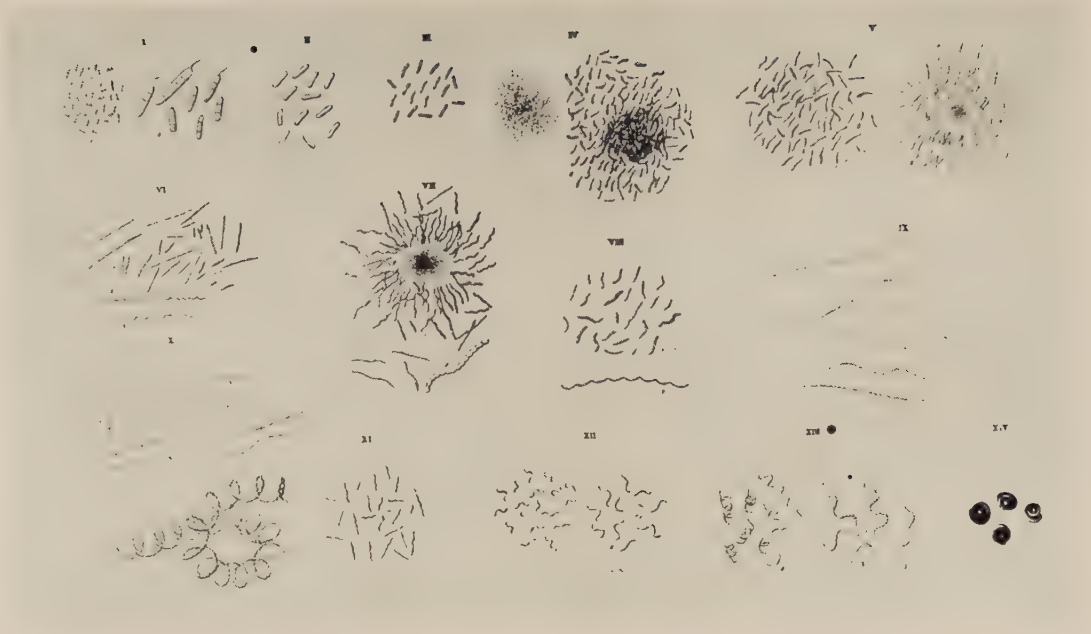

FIG. 16.— Figures of micro-organisms. From Ehrenberg (1838).

[*To face p.* 58.

chief founders of the bacteriological doctrines taught to-day. His classical 'Untersuchungen über Bacterien' (1872, 1875, 1876) will be referred to later.

The term Schizomycetes, universally used to include bacteria to-day, was introduced by the botanist C. Nägeli (1857), but it is of interest to note that Nägeli himself was uncertain whether the forms which he placed in this family were plants, animals or elementary particles.

In the middle of the nineteenth century great advances were made with regard to the study of the life-cycle of certain fungi. It was shown that they exhibit an ' alternation of generations ' or ' pleomorphism ' (see De Bary, 1884). Thus it was conclusively proved that one and the same fungus can appear in several forms, not only in respect of its vegetative, but also of its fructifying organs. In the wave of excitement over this discovery it was believed that pleomorphism was applicable to bacteria and this led to great confusion and error. Before referring to this aspect, however, it is necessary to deal with the discovery of bacterial forms in certain diseases of man and animals.

It was in examination of the blood of sheep dead from anthrax that P. F. O. Rayer (1850), an eminent French physician, reported the occurrence of ' petits corps filiformes, ayant environ le double en longueur d'un globule sanguin. Ces petits corps n'offraient point de mouvements spontanées '. Although Rayer made no other statement concerning the bodies, which appear to have been first seen by Davaine, his assistant (see Davaine, 1875), it was the first dealing with such things in anthrax blood. The bodies of Davaine were independently and much more fully described by a certain Dr. Pollender (1855), of Wipperfurth, who tells us that he had first seen them in 1849 in the blood of anthrax cows. In the spleen he found the rod-like bodies in enormous quantities. They were motionless and in form and arrangement they presented an exact likeness to *Vibrio bacillus* or *V. ambiguus*. Brauell (1857, 1858), a veterinary professor in Dorpat, confirmed the findings of Pollender in the case of a man who had charge of a crematorium for destroying anthrax carcasses. Brauell carried out a great many inoculations with anthrax blood, and showed that the disease was transmissible from man to sheep and horse to horse. He found the rods in the blood of a sheep during life but did not regard them as peculiar to the disease. He stated that whereas in fresh anthrax blood the rods were motionless, they were motile in blood kept a few days. It was, however, chiefly through the long series of researches by Davaine (1863, 1864, 1865) that the subject of anthrax and its rods (' bacteridia ' of Davaine), gained a great prominence. He was ultimately able to state, with considerable precision, his belief that the bacteridia must be the cause of the disease, on the grounds that they are constantly found in anthrax, that anthrax can be transmitted by inoculations in series, and that when bacteridia are absent no anthrax results. Davaine (1868) showed in fact that anthrax can result from the inoculation of so small a quantity as one-millionth of a drop of anthrax blood.

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Davaine's views were subjected to considerable criticism by several workers and particularly by Leplat and Jaillard (1864), two professors in the Val-de-Grace military hospital. They inoculated many rabbits with anthrax blood sent from Chartres to Paris, and were unable to find Davaine's bacteridia, notwithstanding that the fatal disease produced bore a complete resemblance to anthrax. Davaine contended that this disease was not anthrax but a putrefactive condition which was communicable to birds, which true anthrax is not.

Davaine's researches on anthrax did much to further the doctrine of the germ theory of disease, which had to contend with great opposition, although supported by the recent work of Pasteur (1865–70) on the silk-worm disease, *pébrine*. That virus is particulate was also rendered probable, if not certain, by the diffusion experiments of Chauveau (1868) and Burdon Sanderson (1870) on vaccine lymph.

The germ theory was not accepted unopposed, and apart from a vast amount of writing by medical men inadequately equipped for the purpose, many persons of scientific training combated the theory with great ardour. Among the latter may be mentioned Bastian, in all his works from 1872 onward, the English microscopist, Lionel Beale (1870), and especially A. Béchamp, who for many years was the implacable foe of Pasteur, and made it his business to oppose everything which the latter wrote, did, or said (see Béchamp, 1883).

A revival of the early fungoid ætiological period was started by J. H. Salisbury (1862), of Newark, Ohio, who claimed that the causes of many diseases, infective or otherwise, resided in fungi of complex character.

A glance at the bacteriological knowledge about 1865 shows that two views prevailed. According to the one, held by Pasteur, micro-organisms are constant in form and in action. From the technical methods which he used it may be said that Pasteur was unable to prove his point, although, as it turned out, he was correct. His nomenclature was loose, and Cohn (1872) pointed out that he used almost as synonyms, '*végétaux cryptogames microscopiques*', '*animalcules*', '*champignons*', '*infusoires*', '*torulacées*', '*bactéries*', '*vibrioniens*', '*monad*', '*levure*', and other terms equally ill defined. The general word 'microbe' was suggested later by Sédillot (1878). Pasteur had no use for the laws of botanical nomenclature, but whatever he had in mind when he used the above-mentioned terms, he was a firm believer in the constancy of form and action of one and the same micro-organism. In experiments in which it appeared that the forms altered, Pasteur inferred that he was dealing with more than one type of germ. On the other hand the classical work on the polymorphism of fungi led to the belief that a similar state of affairs prevailed among the yeasts and bacteria. This view was developed in Germany, especially by Ernst Hallier (1831–1904), who was Professor of botany in Jena. He was a voluminous writer, and ran a 'Zeitschrift für Parasitenkunde', of which he was almost the only contributor. His reign was brief—a matter of five years—when the edifice which he had erected with so much diligence and

FIG. 17.—Hallier's 'isolation apparatus'. *C* is a flask containing the test substance and connected on the right with the bell jar of an air pump and on the left with a bottle, *B*, with sulphuric acid. This again is connected with a tube, *bw*, filled with cotton-wool. From Hallier (1867).

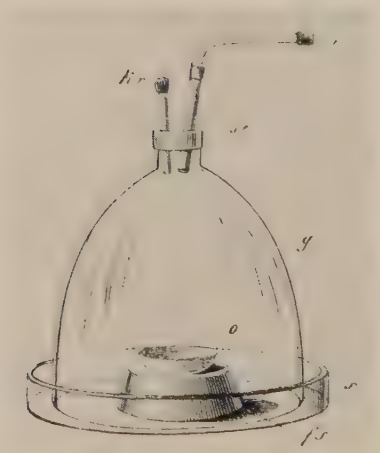

FIG. 18.—Hallier's 'culture apparatus'. *s*, Vessel containing water; *o*, the culture vessel; *g*, a bell-jar connected with an air pump. From Hallier (1867).

To face p. 61.]

with such defective methods, was shaken to its foundations by A. de Bary (1867) one of the greatest mycologists of his time. Hallier's phytopathological works make difficult reading. His main thesis was, however, clear. He held that the microscopic forms of vegetable parasites do not constitute genera and species, but are merely stages (' Morphen ') in the development of more complicated fungi, and that the transformations are brought about through changes in the medium, temperature, and other less important factors. His aim was to study vegetable parasites under conditions altered intentionally, so that their real nature would be unmasked. For this purpose some morbid product was inseminated in different media, and the results were checked by comparison with inseminations kept under other conditions. He devised cultural apparatus of two kinds, the one an ' Isolirapparat ', and the other a ' Culturapparat '. The ' Isolirapparat ' (Fig. 17) was for keeping the culture *isolated* or unopened until the end of the experiment, and was intended to control the ' Culturapparat ' (Fig. 18), in which he followed the alleged morphological changes.

It seems needless to-day to point out that the result in such an apparatus must depend upon what was in the inoculum before it was inseminated in the medium. De Bary put it brutally when he said ' if you wish to prove that A grows out of B it is necessary to prove that A was not present in B before the examination was begun ', and yet it was this very thing that Hallier never seems to have considered, and by its neglect his whole work was wrecked, for both in his culture and his isolation apparatus he found a succession of changes which he regarded as genetically connected. Thus in disease products Hallier constantly saw micrococci, but from his culture apparatus he obtained other things after the medium had been inseminated with material containing micrococci. Cholera stools, for example, yielded a rice fungus, cocci in vaccinia were alleged to grow to the fungus *Eurotium herbariorum*. The micrococci in typhoid stools were said to be a stage of *Rhizopus nigricans*. All the work of Hallier is now forgotten. The moral of it remains, and is best appreciated if we remember the witticism of Brefeld that unless one works with pure cultures ' da kommt nur Unsinn und *Penicillium glaucum* heraus ' !

From the confusion wrought by Salisbury and Hallier with their inadequate methods it is a pleasure to turn to a period characterized by solid advances in knowledge. These advances we owe mainly to Ferdinand Cohn, whose ' Untersuchungen über Bacterien ' will be appreciated at once by the student as the real starting-point of our modern bacteriological ideas. Cohn's researches occupied him laboriously for many years, and he was exceedingly successful in disentangling almost everything that was correct out of a confusion of ideas, and this in what at the time was a very difficult line of study.

Cohn (1872) raised the fundamental question whether bacteria can be arranged in genera and species. This had been accepted as a matter of course by the early systematists like O. F. Müller, Ehrenberg and Dujardin, and Cohn followed in their footsteps, although he realized that it was

necessary to discuss the question. He grounded his belief in the differentiation of bacteria on an accurate study of some of the larger, and especially the spiral, forms, which in his opinion showed a marked constancy irrespective of external conditions. He pointed out, however, that a purely morphological classification is insufficient, as he recognized that bacteria similar or identical in form may differ from each other in their physiological properties and products. Whether these ' form genera ' and ' form species ' are, or are not, natural genera and species, Cohn left for future investigators. The means at his disposal were not sufficient to settle the question. Cohn has often been wrongly represented as the founder of the doctrine that bacteria can be classified on morphological grounds only. No one who has read his works can agree with this interpretation. He realized and expressed himself clearly that one must take into account not the form alone, but all the points of agreement and difference between related forms. He regarded his ' form genera ' as natural genera, but his bacterial species as largely provisional (Cohn, 1876). Cohn gave a very exact account of the constitution of the bacterial cell and suggested the division of bacteria into four groups (Tribus), each of which contained one or more genera. He retained as far as possible the older names but with altered significance. His classification was as follows :

Tribus I. Sphærobacteria.
Genus 1. *Micrococcus* char. emend.
Tribus II. Microbacteria.
Genus 2. *Bacterium* char. emend.
Tribus III. Desmobacteria.
Genus 3. *Bacillus* n.g.
Genus 4. *Vibrio* char. emend.
Tribus IV. Spirobacteria.
Genus 5. *Spirillum* Ehrenberg.
Genus 6. *Spirochæte* Ehrenberg.

Among the round or Sphærobacteria he differentiated chromogenic, zymogenic and pathogenic species, and gave a very full account of all the species as far as they were known at that time. For the chromogenic forms he was indebted to the excellent researches of his co-worker, J. Schroeter (1872). Cohn's pathogenic Sphærobacteria included *Micrococcus vaccinæ* discovered by Cohn himself (1872) and cocci which occur in sepsis and in diphtheritis. Cohn's Microbacteria differed from the sphærobacteria in shape and motility. Two species, *Bacterium termo* and *B. lineola*, were described in great detail. The Desmobacteria or filamentous bacteria of Cohn contained two genera according as the filaments were straight (*Bacillus*) or wavy (*Vibrio*). He gave a very minute account of *Bacillus subtilis* and recognized and figured the spore, which he correctly interpreted as a persisting form of the organism. Davaine's bacteridium of anthrax was placed by Cohn in the genus *Bacillus*. Cohn worked his way through all the known ' bacteria ' and everywhere he added fresh details to our knowledge. He considered that bacteria constitute a fairly

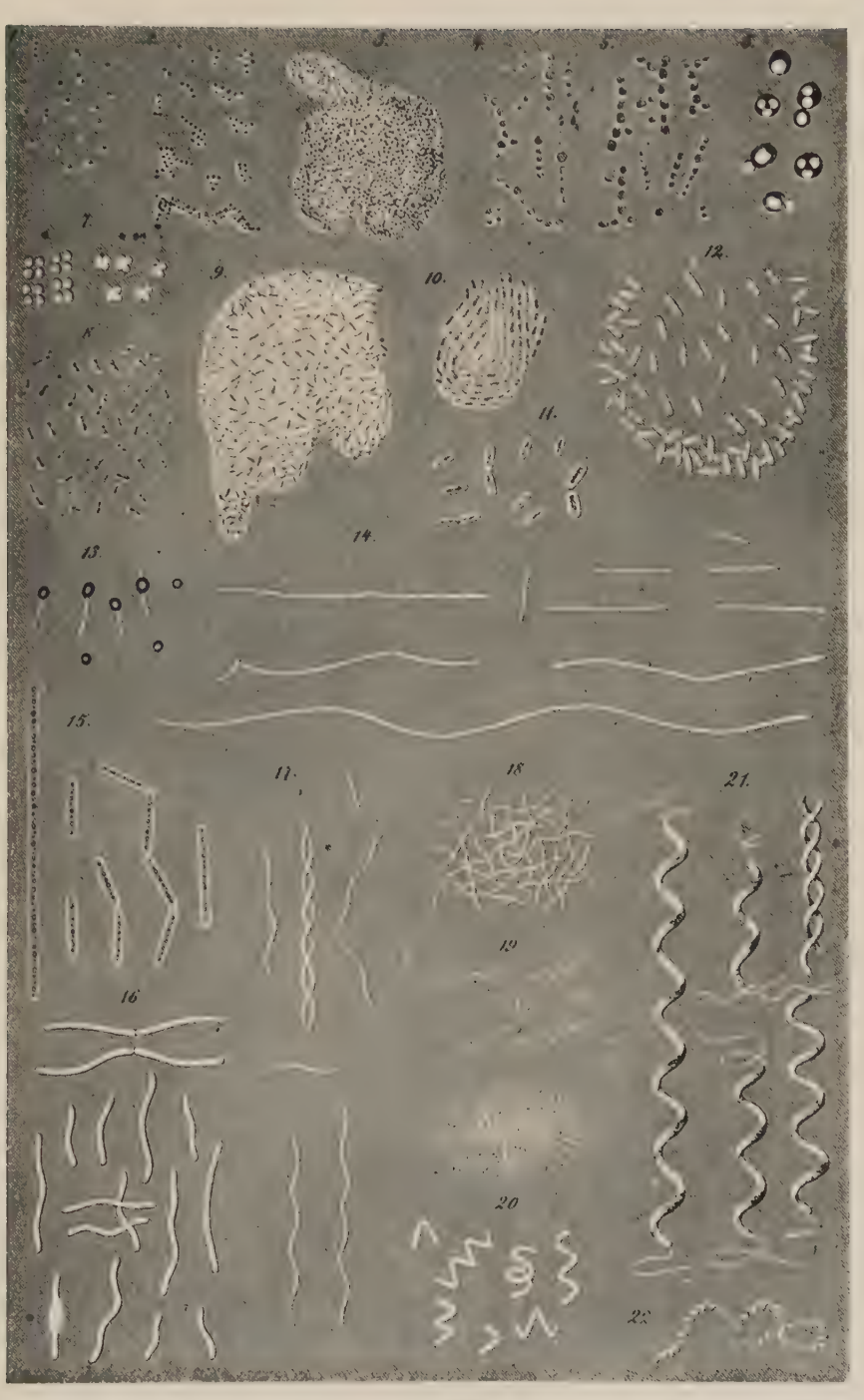

FIG. 19.—Cohn's first plate illustrating bacteria. In Fig. 13 (on the plate) bacterial spores are shown. From Cohn (1872).

[*To face p.* 62.

FIG. 20.—Cohn's second plate illustrating bacteria. From Cohn (1875).

To face p. 63.]

compact group of organisms, which show close affinities with certain of the blue-green algæ. He also carried out many experiments on the metabolism of bacteria, and confirmed much of Pasteur's work. In the course of this part of his investigation he devised the fluid medium since known by his name as ' Cohn's fluid ', which in some respects was superior to that of Pasteur. The plate accompanying Cohn's paper is shown in Fig. 19.

Cohn's views on bacteria were not at once accepted as a correct interpretation of the observed facts, and the pleomorphic theory of bacteria died hard after lingering in the works of Huxley (1870), Lister (1873), Ray Lankester (1873), Klebs (1873), Warming (1875), and especially Billroth (1874), Cienkowski (1877) and Zopf (1879–85).

Of some importance for medical bacteriology at the time was the work of the Viennese surgeon, Billroth (1874), who maintained that all the round and rod-shaped forms of bacteria in putrid materials were but stages of a plant which he called *Coccobacteria septica*. The error into which, as we now know, Billroth fell was occasioned through the neglect of continuous observation. His examinations were made at intervals during which unobserved events took place, so that one bacterium gave the impression of proceeding from another although both were present all the time.

Cohn's second classical paper (1875) pointed out the defects in Billroth's work and contained, in addition, a description of a number of red-coloured bacteria from contaminated water. Unlike Ray Lankester, Cohn held that these were different species rather than forms of one species, and he worked out very carefully the physiology of these interesting ' sulphur bacteria '. He also gave a minute account of spore formation as it occurs in *Bacillus subtilis*. An elaborate classification of Schizophytæ was included by Cohn, whose paper was illustrated by fine plates far in advance of any previously published (Fig. 20). Cohn's third research (1876) dealt with the effect of heat on organic liquids in relation to heterogenesis, and has already been referred to on p. 41.

Nägeli (1877) opposed Cohn's views on the morphological fixity of Schizomycetes and brought forward his ' adaptation ' (Anpassung) theory to the effect that every ' species ' of bacterium appears in several morphological and physiological types, which can be converted the one into the other by external conditions. For example, he alleged that unboiled milk becomes sour, whereas boiled milk becomes bitter. This difference was not due, in his opinion, to two different agents but to the alteration of the medium by the act of boiling. It may appear presumptuous to say so, but the perusal of Nägeli's bacteriological works leaves the impression that he was a verbose philosopher, rather than an accurate observer, whatever he may have been in his other botanical researches. Following Nägeli closely, Hans Buchner (1882) maintained that he had been able to convert *Bacillus subtilis* into *B. anthracis* by shaking at different temperatures.

The botanist, W. Zopf, in a series of works from 1875 to 1885 contended that what he called Cohn's theory had been completely refuted by the work of Nägeli, Billroth, Warming, Lankester and others, and that bacteria were inconstant in form. He considered that it was impossible to place certain micro-organisms in Cohn's genera. In spite of his general statements Zopf, however, classified bacteria into four groups on morphological grounds :

1. Coccaceæ.—Known only in globular form.
2. Bactericeæ.—Consisting of coccal, rod and thread forms, but without distinction of base and apex.
3. Leptotricheæ.—Cocci, rods and thread forms, the latter with base and apex distinction.
4. Cladotricheæ.—Cocci, rods, spirals, and showing dichotomy.

Zopf's views were widely disseminated by the publication of his book ' Die Spaltpilze ' (1885), and again raised for a time active discussion on the variability of bacteria. Pleomorphism was also alleged to occur by Metchnikoff (1888), but rejected by Winogradsky (1887, 1888) from his studies on the sulphur bacteria. The term ' pleomorphism ' has been frequently used to denote different things. Thus it has been employed for the slight and inconstant changes in bacterial form seen under different conditions of nutrition. It has also been used to denote the so-called ' developmental cycles ' of limited range which some bacteria exhibit. In these senses ' pleomorphism ' is not what the name was introduced to represent. The pleomorphic doctrine of Zopf and others that cocci change to rods or spirals is no longer accepted as correct.

While the botanists were wrangling on the subject of morphological constancy of bacteria, the medical bacteriologists were content to record the presence of bacteria in morbid processes. From 1867 to 1877 many observations were made which showed that small bodies generally designated ' micrococci ' occurred in diphtheritis (Buhl, 1867), endocarditis (Winge, 1870 ; Eberth, 1872), and in septic and putrid infections (Leyden and Jaffe, 1867 ; Klebs, 1873 ; Weigert, 1876). These early observations and many others of a like kind did a good deal to advance the germ theory, but now possess only historic interest.

Of great importance for the doctrine of specific pathogenic germs were the theoretical considerations brought forward by Weigert (1875). He discussed the question whether the objects called bacteria were really such, or whether they were degeneration products of the tissues. He dealt with the relation of bacteria to the morbid processes or products, and the problem whether such processes or products emanated from the bacteria or from other sources. Finally he discussed the question whether the something that makes bacteria pathogenic is a vital bacterial product, or merely attached to the bacteria. He endeavoured to show that bacteria stain differently from degenerative products. In the case of smallpox, with which he was specially dealing, he showed that the necrotic areas found in the early stages of the disease stand in close relation to the central

bacterial foci. The bacteria are primary and the necrobiotic process is secondary to their action. The idea that harmless bacteria can migrate to a focus already damaged by an independent chemical poison was regarded by Weigert as improbable, for the reason that foci are not found in variola before the bacteria make their appearance. The fact that bacteria may grow in the body without producing symptoms proved nothing, in Weigert's opinion, unless it could be shown that all bacteria were endowed with the same properties, or at any rate unless, in two instances the subject of the same comparison, the two bacteria were the same. There was no reason to suppose that because bacteria were microscopic they were necessarily equivalent. Although Weigert's work was only in part experimental, his theoretical conclusions carried great weight, and stimulated a further interest in the germ theory of disease. Towards the end of the seventies it may be said that this theory had made rapid headway and had gained a firm footing as a medical doctrine. Bacteriology then began to emerge as a definite science. People no longer spoke of 'the' bacteria but of different bacteria. In this development both botanists and medical men played their respective parts. Among the former must be mentioned as of special merit, Ferdinand Cohn, most of whose observations and conclusions have remained unassailable to-day. It is true that some of the questions which he raised are still in the melting-pot, and nowhere is this so evident as in the classification of bacteria, for none of the attempts of Zopf (1885), Schroeter (1889), De Toni and Trevisan (1889), Hueppe (1886), Marshall Ward (1892), Alfred Fischer (1895), Migula (1897), Lehmann and Neumann (1896–1927), down to Orla Jensen (1908, 1909), Winslow and Broadhurst (1917), and the Society of American Bacteriologists (Bergey, 1923), has been entirely acceptable to bacteriologists.

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Development of Bacteriological Technique : Researches of Robert Koch and Pasteur.

The prolonged researches of Davaine upon anthrax had rendered it highly probable that this disease was caused by the rod-shaped micro-organism which he called ' bacteridium '. This view was further supported by the experiments of Eberth (1872), who allowed anthrax blood diluted with water to settle. Inoculation of the supernatant fluid was found to be without effect, whereas the sediment was capable of producing anthrax. Davaine (1870[1 & 2]) found that anthrax may be communicated by flies, but in 1875 there still remained a number of questions which the theories of the period could not explain. It was at this time that a new investigator appeared on the scene to throw a flood of light on the still unsolved mysteries of anthrax. This was Robert Koch (1843–1910), who at the time was actually a country doctor and district physician at Wollstein—a small town in Posen. We are introduced to Koch by Ferdinand Cohn (1876), who tells us that he had received a letter (dated 22 April, 1876) from him, to the effect that after prolonged effort he had discovered the complete life history of the anthrax bacillus, and that he was prepared to demonstrate his results to Cohn in Breslau. The meeting took place in Cohn's Institute on April 30, 1876, and lasted three days, at the end of which time Koch had convinced his audience of his discovery. The occasion is historic, and among those present at the demonstration were Cohnheim and his assistant Weigert, Auerbach (of plexus fame), Moritz Traube, and Eidam, a co-worker of Cohn.

As ' Kreisphysikus ' Koch (1876) had occasion to study anthrax in domestic animals, and had come to the conclusion that many of the facts suggested that the bacillus was capable of spore formation. Placing minute fragments of fresh anthrax spleens in drops of sterile aqueous humour or blood-serum he set himself to watch, hour after hour, what took place. He saw the bacilli grow into long filaments, especially at the edge of the cover-slip, and, as he watched, he saw rounded and oval granular bodies appear inside the filaments. The bodies became clearer and stood out as prominent objects. He realized that they were spores, which had not been seen before. In slides there was every stage from the bacteridium of Davaine to the fully formed spore. On a primitive warm stage at different temperatures he determined accurately the optimal thermal conditions for spore formation. By ingenious methods he disposed of the possible criticism that the spores belonged to some other micro-organism which had wandered on to the slide from without, and he succeeded in showing that under suitable conditions the spores grew again into anthrax rods. Koch described and figured the germination of anthrax spores, and showed that they were highly resistant to injurious influences. He grasped, at once, the significance of this fact from the epidemiological point of view. Using primitive methods he showed that anthrax was transmissible from mouse to mouse in a series of twenty generations, and that the

lesions in each member of the series were identical. He also worked out the dissemination of the bacilli in different animals.

Koch (1876) showed that anthrax and experimental septicæmia differ from each other, although in putrid infusions of vitreous humour he found bacilli almost identical in form and spore formation with anthrax bacilli. Inoculation of such bacilli did not result in anthrax. From facts like these Koch concluded that ' only one kind of bacillus is in position to induce the specific morbid process, whereas other schizophytes are not, or, if pathogenic, they act in a manner different from anthrax '. Koch thus clearly enunciated the idea of a specific virus. In the case of anthrax in frogs he gave an accurate description and figured the changes subsequently known as phagocytosis. The plate accompanying Koch's (1876) classic research is shown in Fig. 21.

Koch's discoveries on anthrax, published under the ægis of Cohn, were at once recognized as from the hand of a master. Through the exertions of Cohn and Cohnheim he was able to free himself from the claims of practice, and enter upon that long career in which he was acknowledged as one of the greatest exponents of bacteriology. Koch's anthrax research was born complete, and no substantial changes have since been made in it.

In the meantime Pasteur had wished to study anthrax, but hesitated, as he says, because he was ' étranger aux connaissances médicales et vétérinaires '. He sought for a suitable collaborator and found one in Joubert, a professor in the College Rollin. Pasteur and Joubert (1877[1]) then began to investigate anthrax *de novo*. Although their work added relatively little to the knowledge which Koch had gained in his anthrax studies, it helped to fix the conviction that anthrax is a bacterial disease specific or special in its characters. The study of bacteria now proceeded along two separate lines. The one was taken by Koch, and had for its object the examination and cultivation of bacteria. The other was taken by the French, led by Pasteur, who turned to the experimental analysis of the manner in which infective diseases are produced and recovered from.

There was probably a reason why Koch pursued the course he did. He was working at first in a very small way, and had to be economical in his animal experiments. He concentrated, therefore, on the technical methods of bacterial examination, and his efforts yielded a wonderful harvest of results, as will soon be apparent. The chief methods which he developed were in connection with staining and cultivation of bacteria.

The staining of bacteria followed on the ordinary staining methods of histology. In the case of the latter carmine was recommended by Goeppert and Cohn (1849) and Gerlach (1858), and hæmatoxylin by Waldeyer (1863) and Böhmer (1865). The preparation of dyes from aniline and coal-tar began about 1856, and substances like mauvein and fuchsine soon came into the market. H. Hoffmann (1869) was the first to attempt to stain bacteria, and he used carmine or fuchsine for the purpose. Weigert (1871) found that carmine can stain cocci, but the staining of bacteria as an art really dates from his observations in 1875, when he showed that

FIG. 21.—Plate accompanying Koch's first paper on anthrax. From Koch (1876)

[*To face p* 68

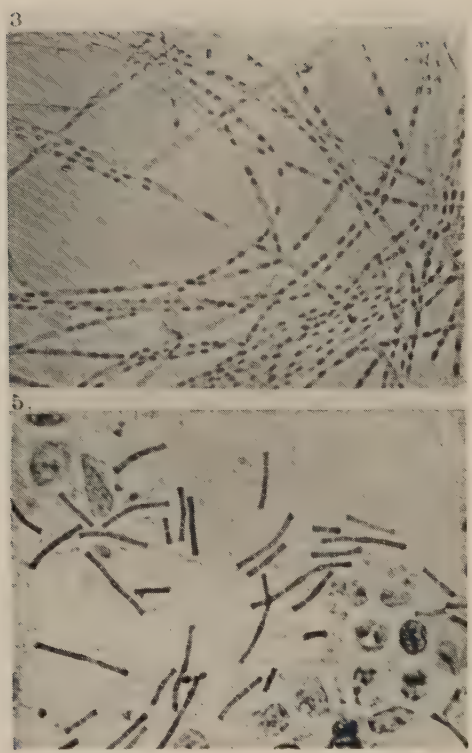


FIG. 22.—Two photographs of Bacillus anthracis taken by Koch. From Koch (1877).

To face p. 69.]

methyl violet can be successfully used to reveal cocci in animal tissues. Salomonsen (1877) stained bacteria by a watery solution of fuchsine.

In his second bacteriological research, Koch (1877) greatly improved the methods of bacterial staining and laid the foundation of the technique employed to-day. Realizing the importance of getting the bacteria into a motionless state he prepared thin films on cover-glasses and dried them. To his surprise the form of the bacteria remained unchanged. He then fixed the films in alcohol and applied various stains, the most successful of which were methyl violet 5 B, fuchsine, and aniline brown (Neubraun). The stained preparations were finally mounted in potassium acetate solution or canada balsam. These preparations were better than any that had been seen before Koch's time, and many of them were reproduced in an excellent series of photographs taken by Koch using sunlight as an illuminant (Fig. 22). He also succeeded in staining bacterial cilia. These had previously been figured by Ehrenberg (1838) in *B. triloculare* (see Fig. 16) and by Cohn (1872) in *Spirillum volutans* (see Fig. 19), but by means of logwood extract and subsequent treatment with chromic acid, Koch was the first to obtain them in the stained state. From then onwards bacterial staining methods were rapidly perfected. Much of this had for a basis the remarkable work of Ehrlich on the staining of specific granules in leucocytes by aniline dyes. This began in 1877—at a time when he was only 23 years of age—and lasted till 1881 when his blood work was practically finished.

Bismarck brown was introduced as a stain by Weigert (1878) and methylene blue by Ehrlich (1881). On account of its properties, the latter dye soon came to be extensively used in bacteriology and was the one by which Koch discovered the tubercle bacillus in 1882. He found that when an alkali was added to the solution of methylene blue, the dye is able to penetrate into the tubercle bacillus, and remain there, in spite of subsequent treatment with vesuvin or Bismarck brown. The addition of alkali to methylene blue was also the principle of 'Loeffler's blue' (1884) which is used to-day. It was in 1881 that Koch employed heat as a fixative for bacterial films. On the day after he announced his discovery of the tubercle bacillus (March 24, 1882), Ehrlich (1882) succeeded in improving Koch's method and shortly afterwards published the essence of the method that is universally known as Ziehl-Neelsen's process. Instead of potassium hydrate as the alkali, Ehrlich used aniline as an addition to methyl violet or fuchsine. Ehrlich was led to the employment of aniline by observing that preparations of impure violet, such as gentian violet, stain better than pure, and he regarded the enhancing element in the impure dye as being probably aniline. This substance shaken with water and filtered yielded the so-called aniline-water solutions, which Ehrlich then saturated with violet or fuchsine in alcoholic solution. By these the tubercle bacillus was found to be rapidly and deeply stained, and the colour was found by Ehrlich to be 'acid-fast' in that it could not be discharged even by nitric acid in a concentration of 30 per cent. in water. As a counterstain

Ehrlich used vesuvin or methylene blue, according as the bacillus was primarily stained violet or magenta. The use of carbolic acid instead of aniline water was the contribution of Ziehl, and the use of sulphuric instead of nitric acid was the contribution of Neelsen (see Johne, 1885). The merit of first staining tubercle bacilli by the methods used to-day was solely Ehrlich's, the modifications of Ziehl and Neelsen being trivial. Ehrlich's aniline-water solution of methyl violet was also employed by Christian Gram—a Dane working in Berlin—in the development of the universally known 'Gram's process' of staining. What Gram (1884) found was that if bacteria are stained by Ehrlich's aniline-water gentian violet solution, then treated with Lugol's solution of iodine in potassium iodide, and finally placed in alcohol, the violet colour is discharged from certain bacteria but retained in others. This Gram's method—for Gram invented no new solution—has been slightly modified by many workers, but in its essence remains unaltered, and is one of the most widely practised methods for the differential staining of bacteria.

Special methods were also discovered for staining spores, cilia, and capsules, and the number of these technical processes to-day is very large. They are for the most part trivial modifications of the original processes laid down by the master stainers, Weigert, Koch and Ehrlich.

The idea of bacterial species as held by Ehrenberg, Cohn and Schroeter early led to the belief that it might be possible to obtain pure cultures, i.e. growths of one particular kind of micro-organism unmixed with others. This was supported by the observations of Pasteur (1857), that the lactic fermentation is due to a characteristic organism which can be grown in transparent liquid media. This was the method almost invariably used by Pasteur, and attempts were made to obtain the most suitable nutritive media. A small portion of the material to be cultivated was transferred by a sterile instrument to a sterilized medium in bulk, and the latter was said to have been inoculated. When growth had taken place a minute quantity of it was inoculated into fresh medium, and so on in series. It was early noted, however, that media vary much in their nutritive properties, and efforts were directed to secure universal media which would answer the requirements of all bacteria. Pasteur (1861[2]) recommended a fluid consisting of water, 100 parts, pure candy sugar, 10 parts, ammonium tartrate, 0·2–0·5 parts, and the ashes of yeast, 0·1 part. This 'Pasteur's fluid' was very extensively used, as also its modification in the form of Cohn's fluid (1872). In addition to these, various aqueous extracts and decoctions of vegetable substances (hay, turnips, carrots) were also used, as well as milk and urine. The growths on such media were often feeble, and an elaborate research was undertaken by Nägeli (1882) to determine the nutritive requirements of bacteria. He examined the effect of the presence of carbohydrates and proteins in the nutritive fluid, and found that sugars and peptone were the best. Miquel (1883) suggested the use of Liebig's extract of meat or a bouillon made from meat direct. The basis of the common nutrient broth used

everywhere to-day was worked out by Loeffler (1881) in connection with his successful cultivation of the bacillus of mouse septicæmia. He grew this micro-organism in a medium consisting of meat infusion to which were added peptone, 1 per cent., and common salt, 0·6 per cent., the reaction of the final product being made faintly alkaline by sodium phosphate.

The first attempts to obtain separate cultures of pathogenic bacteria were those of E. Klebs (1873) by what he called the ' fractional method '— not a very happy name. The principle was to introduce into a sterile medium a small portion of the material to be cultivated, by means of a capillary pipette. After vegetation had occurred a minute fraction or quantity of the growth was placed in a second quantity of medium and so on in series. It was hoped that any contamination originally present would be eliminated, and that the organism present in the greatest quantity would ultimately dominate the situation and show itself as a pure cultivation. Klebs is reticent on his results, but it is practically certain that he never obtained pure cultures.

Better results were obtained on opaque solid media. This started from the observations on blood-red stains which at various times occurred sporadically or in epidemics on articles of diet. It was in an extensive outbreak in 1819, in Legnaro, near Padua, that Vincenzo Sette (1824) showed that what had been regarded as a portent was really something living, and that it grew on bread or polenta to which it had been transferred. Sette called the living agent *Zoogalactina imetrofa* but it was later called *Micrococcus prodigiosus*. Attempts to grow micro-organisms other than those which developed colour were made by H. Hoffmann (1865), who employed a corked tube half-filled with water, which was then boiled. A piece of potato or bread was then introduced into the tube, which was again boiled. The excess of water being poured away a trace of yeast was applied to the surface of the medium by a long needle. After some days, growth occurred and was found to be a mould. Hoffmann's apparatus was described as a ' vapour tube ' (' Dunstrohr ') and its object was to exclude aerial germs and to allow the inoculum to vegetate on a moist solid surface. Brefeld (1874) said of the method that it was on a par with a man, already soaked, putting on a rain-coat to protect himself against the last few drops of rain.

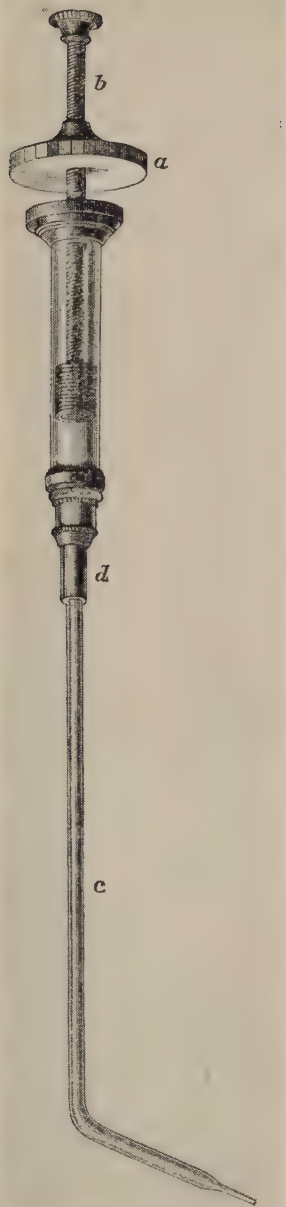
Solid media were used with great advantage by J. Schroeter (1872) in his classical work on chromogenic bacteria. Potato, starch paste, flour, egg albumin and meat were all utilized by him, and on them he obtained bacterial growths, red, blue, green, orange, yellow, brown or violet in colour. In the different masses or colonies the bacteria were different, whereas they were constant and alike in one and the same colony. Schroeter no doubt was dealing with pure cultures, but there were difficulties when one had to deal with forms which were practically the same colour as the medium itself.

Potato as a medium was also employed by Koch (1881[1]), who, however, greatly improved the technique of its preparation. This he did by soaking

the cleansed potato in a solution of corrosive sublimate (1 : 1,000). The potato was then sterilized in steam and finally split in two by a sterile knife. The two halves were allowed to fall apart inside a covered glass vessel which had been previously sterilized. The cut surface of the potato was finally inoculated in streaks with the bacterial material.

Klebs (1873) attempted to get cultures by inoculating very minute quantities of bacterial material in fluids, which were then placed in Recklinghausen chambers. Difficulties were experienced with watery fluids, and to render the medium stiff Klebs added isinglass. Gelatin had been used as a medium by Vittadini (1852) in his cultivation of the fungus of muscardine. The addition of gelatin or decoction of carragheen to liquid media was also suggested by Oscar Brefeld (1872). To this great mycologist must be given the credit of laying down with precision the principles which must be followed to obtain pure cultures. His researches were presented in a series of volumes from 1872 onwards. These magnificent works, profusely and splendidly illustrated by Brefeld himself, dealt with large groups of fungi, but of special interest was the series entitled 'Botanische Untersuchungen über Schimmelpilze' (1872, 1874, 1881). In addition he published a number of papers, the most concise on the subject of cultivation being that published in 1875. The new principles he inculcated were (1) that the insemination of a sterile nutrient medium should be made from one single fungal spore, (2) the medium should be transparent and should yield optimal growth for the fungus under observation, and (3) the culture throughout should be completely protected against external contamination. Brefeld's technique, admirable as it was for relatively large objects like the spores of moulds, was found to be very difficult when applied to minute objects like single bacteria. Other methods were suggested and among them that of dilution. A fluid containing a number of bacteria was diluted down with some sterile fluid medium in the hope that ultimately a growth would be obtained which had taken its origin from a single microbe. Nägeli and Schwendener (1877) denied that this method could be successful, but Lister (1878) actually succeeded, and was perhaps the first to obtain a really pure culture.

By means of a specially constructed syringe (Fig. 23) having a nut, *a*, revolving in a fine screw on the piston rod, *b*, each degree was made to correspond to the delivery of 1/100 of a minim. The nozzle of glass tube, *c*, was screwed on the body of the syringe by means of a brass adapter, *d*. The bacterial fluid to be examined was diluted down one million times, so that 1/100 of a minim contained, presumably, one single bacterium. This quantity was delivered from the syringe into each of five liqueur glasses (Fig. 24) containing sterile milk. Only one showed growth, and the milk soured. Examination disclosed that only one kind of bacterium was present, which Lister called *Bacterium lactis*. It bred true to type in successive cultures and was, in fact, a pure culture. The dilution method was greatly improved by E. C. Hansen, working in the Carlsberg brewery



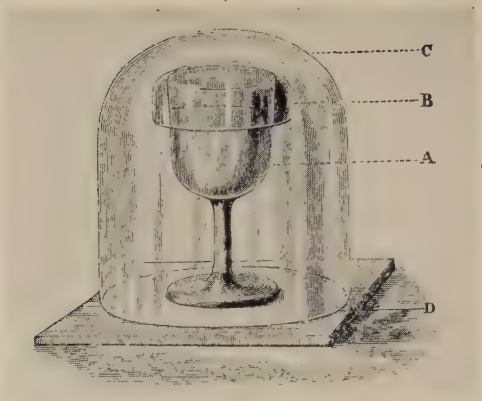


FIG. 24.

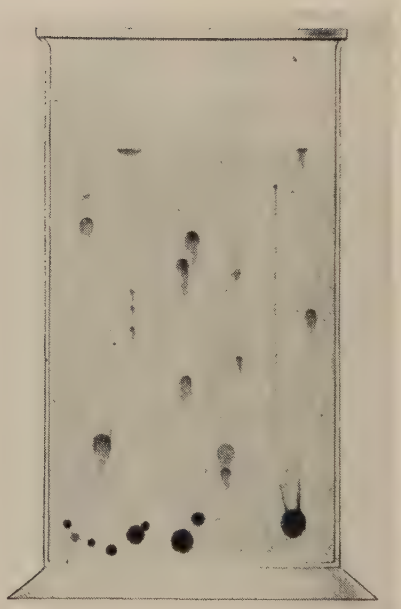


FIG. 23.

FIG. 25.

FIG. 23.—Lister's syringe. From Lister (1878).

FIG. 24.—Lister's liqueur culture glass. From Lister (1878).

FIG. 25.—Glass cylinder with putrefying blood showing dark spots of bacterial masses. From Salomonsen (1877).

FIG. 26.—Salomonsen's capillary culture tube filled with putrid blood. At *b*, *c*, *d*, *e*, *f* and *g* colonies of bacteria presenting different appearances. From Salomonsen (1877).

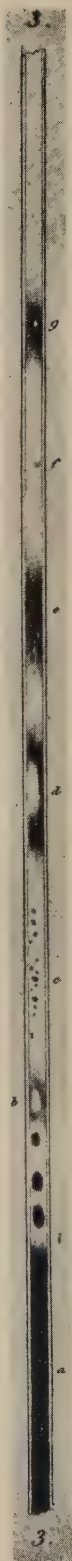


FIG. 26.

To face p. 73.]

in Copenhagen (Hansen, 1883; Pedersen, 1878). For the cultivation of pure yeasts Hansen's methods were of great importance.

The mechanical separation of bacteria in putrid blood was employed by C. J. Salomonsen (1876, 1877) to obtain individual colonies. Having observed that dark spots (Fig. 25) appear in glasses containing putrid blood, he found that they were really pure cultures of different kinds of bacteria, and he isolated them in a more perfect manner by aspirating putrid blood into long capillary glass tubes (50–60 cm. × 0·5–1·0 mm.) which he was able to place under the microscope for observation (Fig. 26). Breaking the tubes where isolated spots occurred, he found that each spot was in fact a colony of one kind of bacterium. No spots occurred in blood taken aseptically from the vessels of healthy living animals. Salomonsen's method had limited application and was given up later on.

Another early method of obtaining what were regarded as pure growths consisted of inoculating susceptible animals with some particular kind of infected material. This was the method of Coze and Feltz (1866) and Davaine (1872) in connection with their studies on septicæmia. The method of ' passage ' was also employed by Koch in the case of anthrax (1876) and traumatic infective diseases (1878). Koch (1878) regarded the successive transmission of artificial infective diseases as ' the best and surest method of pure cultivation ', and he showed that the animal body can be utilized to separate two kinds of bacteria otherwise inseparable. From putrefying blood the only organisms which survived in the bodies of white mice were a small bacillus and a chain coccus. The two in association could be transmitted in series from house-mouse to house-mouse. A separation, however, was possible when the blood from an infected house-mouse containing both bacteria was injected into a field-mouse. In the latter the bacillus disappeared whereas the coccus persisted and could be transmitted pure in series of field-mice.

The method of propagating infective diseases by inoculation has been widely practised and may be, and indeed usually is, the only one which can be used when the virus cannot be cultivated *in vitro*. It is the method of propagating the unknown viruses of vaccinia, rabies, foot-and-mouth disease, fowl plague, swine-fever and similar diseases. Presumably, frequent transferences by passage lead to purification of the virus, although this cannot as yet be definitely proved.

The necessity of obtaining pure cultures of bacteria *in vitro* was early realized by Koch (1881[1]), and he attempted to achieve this end by a simple and consistently successful method. Grasping all the technical difficulties and necessities of the problem he tried to obtain a good medium which was at once *sterile*, *transparent* and *solid*. After many trials he arrived at the conclusion that it was scarcely possible to compound a kind of universal medium which would be of equal nutritive value to all bacteria. He, therefore, concentrated his efforts on solidifying well-tried nutritive fluid media with some clear substance, and recommended for this purpose a $2\frac{1}{2}$ to 5 per cent. of gelatin. The product was referred to

as ' nutrient gelatin ' (Nährgelatine). The essential ingredient in the nutritive fluid was 1 per cent. of meat extract, and he gave exact details how the nutrient gelatin should be prepared. For use he poured the melted gelatin on sterile glass slides, and allowed it to set under cover of a bell-jar, to avoid external contaminations. The actual insemination of the gelatin was made by taking a minimal quantity of the inoculum by means of a sterilized needle or platinum wire, and drawing it in several cross lines rapidly and lightly over the surface of the jelly. Different colonies of bacteria soon made their appearance, and traces of them were transferred to test-tubes plugged with cotton-wool, and containing sterile nutrient gelatin which had been set in an upright or slanted position. In this simple way Koch had solved a problem which three years previously he had deemed to be almost incapable of solution. He soon gained world-wide fame by the demonstration of his method and results in the Physiological Laboratory in King's College, London, at the International Medical Congress in 1881. The audience at his demonstration included Pasteur, Lister, Sanderson and Chauveau. Lord Lister once told me that the moment Pasteur saw Koch's cultures he turned to Koch, whom he had not met previously, and said, ' C'est un grand progrès, Monsieur ! '—a statement which history soon verified. It was in the same Congress that the English heterogenist, Charlton Bastian, reiterated all his old views on spontaneous generation with his accustomed fervour. Addressing him, Pasteur dramatically raised his hands to heaven and exclaimed, ' Mon Dieu, mon Dieu ! est-ce que nous sommes encore là ? Mais, mon Dieu ! Cet n'est pas possible ! '

Continuing his researches with all the assiduity of his race Koch now made many discoveries by his method and strove to make it perfect. The poured plate method was published in 1883, and was originally recommended for isolating pure bacterial cultures from fluids such as water. Instead of streaking the bacterial material on gelatin already solidified Koch now mixed the inoculum with melted gelatin, and, having well distributed the bacteria by shaking, he poured the gelatin, still fluid, on cold sterile glass plates. The separation of colonies was much better by this process, which became the method of election. By this means a very large number of bacteria were obtained pure from all sorts of material—air, water, soil and organic fluids. Bacteriology grew at an enormous rate, and from all parts of the world students flocked to Berlin to learn under Koch the new technique which appeared to open up inexhaustible fields of research.

The claims which Koch put forward for his method were stated by him thus : ' The peculiarity of my method is that it supplies a firm and where possible a transparent pabulum ; that its composition can be varied to any extent and suited to the organisms under observation ; that all precautions against the possibility of later contamination are rendered superfluous ; that subsequent cultivation can be carried out by a large number of single cultures of which only those cultures which remain pure

are employed for further cultivation, and that, finally, a constant control over the state of the culture can be obtained by the use of the microscope '. The subsequent history of bacteriological technique has verified to the letter all that Koch claimed for his method, which after nearly half a century has sustained no change in principle. The nutrient basis of the medium has been varied to an endless extent by the addition of substances intended to enhance the nutritive value or to exhibit some particular chemical reaction caused by the growth of the micro-organism under examination. The titration of the medium has been simplified and standardized by the methods of physical chemistry. The solidifying element—gelatin—in Koch's original method has undergone only one or two important changes. One disadvantage of gelatin was soon experienced, viz., that it does not remain solid at body temperature, and that it is liquefied by certain bacteria. The substance agar-agar was applied to remedy these defects, and was introduced into bacteriological technique at the suggestion of Frau Hesse, the wife of W. Hesse, one of Koch's early co-workers, who studied bacteria of the air and who experienced difficulties owing to the liquefaction of gelatin plates. In the bacteriological literature agar-agar is first mentioned by Koch (1882) in his paper on tuberculosis.

Blood-serum stiffened by heat also yields a fairly clear medium of great nutritive value, and was introduced by Koch (1882) for cultivating the tubercle bacillus. Koch's gelatin or agar plates were employed in their original form for some time, but were replaced when R. J. Petri (1887), an assistant of Koch, introduced what he called a ' slight modification of the process '. This consisted in pouring the melted medium into a glass dish provided with a cover, instead of on a flat glass plate. The method provided for a repeated examination of the plate without the risk of aerial contamination at any stage of the process. Petri's ' dish ' or ' plate ' was destined to replace Koch's plates immediately and permanently, and his technique has not been altered down to the present time.

Filtration of bacterial liquids. The methods of bacterial filtration date from the experiments of Tiegel (1871), who filtered anthrax fluids through a porous cell of unburnt clay by the aid of a Bunsen air pump. Eberth (1872) also used this method in his experiments on diphtheritis. Pasteur and Joubert (1877[1 & 2]) employed plaster of Paris to separate anthrax bacilli from fluids containing them. Miquel and Benoist (1881) used a flask with a long neck, the lower part of which was packed with asbestos covered with plaster of Paris. In 1884 Chamberland gave a short account of a filtering apparatus of candle shape (bougie filtrante), and composed of unglazed porcelain. This material had apparently come into use in Pasteur's laboratory, and the filter was improved by Chamberland, who added an end-piece of nipple shape and composed of glazed porcelain fused to the body of the bougie. This is the ordinary bacterial filter so widely known as ' Chamberland's filter, système Pasteur '. In 1891 Nordtmeyer introduced a new filter medium made of

the compressed infusorial earth known as *Kieselguhr*. Attention was directed to the filtering capabilities of this substance by the fact that the ground-water in the Kieselguhr mine in Unterluss (Hannover) was of a clear blue colour. The filter of Kieselguhr was called Berkefeld from the name of the owner of the aforesaid mine. Other filters have been used differing in shape and size, but the Chamberland and Berkefeld bougies continue to be extensively used.

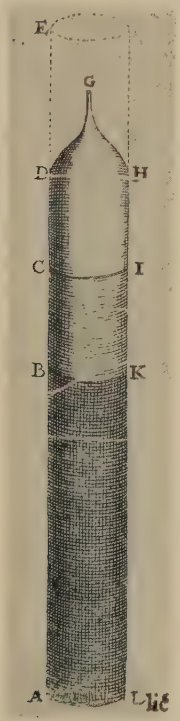


FIG. 27.—Leeuwenhoek's tube for growing animalcules in a rarified atmosphere. From Leeuwenhoek (1695).

Anaerobiosis and methods of anaerobic culture. Attention was directed by Pasteur (1861[1]) to the existence of micro-organisms capable of living in the absence of oxygen. His observations of this date dealt especially with the so-called 'butyric ferment'. That animalcules can exist without air or at any rate in a highly rarified atmosphere was shown by van Leeuwenhoek in 1680. In his 32nd letter (14 June, 1680) to the Royal Society he described and figured a glass tube sealed at one end (Fig. 27), and filled to the level BK with powdered pepper, and to the level CI with fresh rain-water. By the aid of a flame, the open end of the tube was constricted and drawn to point G. A control glass tube was left open. In the sealed tube animalcules developed, although the contained air must have been in minimal quantity. Beijerinck (1913), who repeated Leeuwenhoek's experiment, considered it was the first recorded one on anaerobic bacteria, for he found in the sealed tube *Bacterium coli*, *Azotobacter* and *Amylobacter saccharobutyricum*, and he inferred that Leeuwenhoek's small animalcules must have been of bacterial nature. Spallanzani (1776) also saw animalcules developed in a high vacuum and that they could live and move for 16 days. These observations were, however, overlooked and anaerobiosis was rediscovered by Pasteur (1861[1]). In a later publication (1861[2]) he showed that yeast, grown over mercury, in the absence of air can bring about typical vinous fermentation, and still later (1861[3]), he saw fermentation of calcium tartrate by a micro-organism which was capable of growing under a layer of oil. Pasteur (1863) introduced the terms 'aerobes' and 'anaerobes' to indicate micro-organisms which live with or without free oxygen. Pasteur and Joubert (1877[2]) discovered an anaerobe which produced disease. The microbe in the form of long filaments occurred in anthrax blood which had undergone putrefaction, and was called the septic vibrio (*vibrion septique*). Brefeld (1874) opposed Pasteur's statement that yeast can ferment under anaerobic conditions, but Paschutin (1874) showed that frog's muscle may putrefy, even if kept in atmospheres of hydrogen, nitrogen, carbonic oxide, or even coal-gas. Hufner (1876) devised an

ingenious experiment in which putrid material was brought into contact with boiled fibrin and water in a vacuum. Gunning (1877, 1878, 1879), a Dutch chemist, was of opinion that strict anaerobiosis was not obtained by the methods ordinarily in use, and he clearly showed the great difficulty of freeing apparatus from small quantities of oxygen. On the other hand, Nencki (1879) believed that bacterial growths can take place in perfectly anaerobic conditions. With regard to yeast, Cochin (1880) showed that in the continued absence of air the power of reproduction was lost. Hoppe-Seyler (1884) regarded Pasteur's idea of life without air as highly improbable, whereas Lachowicz and Nencki (1884) saw putrefaction occurring under conditions in which no free oxygen could be detected even by the most delicate tests. It was about this time that technical methods of cultivating anaerobes began to be employed, and of these may be mentioned the apparatus of Hans Buchner (1885, 1888), Hauser (1885), W. and R. Hesse (1885), Liborius (1886), Gruber (1887), Vignal (1887), C. Fraenkel (1888), Roux (1887), Kitasato (1889). From this time down to the present day there has been one continuous stream of anaerobic apparatus of every shape, size and design, all based on permutations and combinations of a few elementary principles.

Sterilization. The necessity for sterile apparatus and media was early appreciated, and the methods for their production were the outcome of the researches of Spallanzani, Schwann, Pasteur, Schröder and v. Dusch, Bastian and Tyndall. Their efforts have already been considered. The modern application of the principles they inculcated developed particularly out of the work of Koch and Wolffhügel (1881). Koch, Gaffky and Loeffler (1881) determined the sterilizing limitations of steam at 100° C. in an exact manner. The important principle of discontinuous heating was the contribution of Tyndall. The extraordinary sterilizing effect of superheated steam came especially from Pasteur's observations of the effect of immersing hermetically sealed flasks in a calcium chloride bath heated above 100° C. The technical disadvantages of his method were obviated when the flasks were merely plugged with cotton-wool, and the sterilizing vessel was closed on the principle of Denys Papin's ' digester or engine for softening bones ' (1681). Modern apparatus of this kind are called ' autoclaves ', of which the smaller laboratory forms were made very convenient by the Paris engineering firm of Wiesnegg under the name of ' Chamberland's autoclave ' about 1884.

The simple method of pure cultivation which Robert Koch (1881[1]) had invented yielded within a few years an amazing harvest of results which fell almost exclusively to German bacteriologists. In addition to a very large number of saprophytic bacteria which were isolated pure from every conceivable source, the ætiological agents of many human and animal diseases were revealed for the first time, and as a result led to an entire reconstruction of ideas on causation of disease. Prior to Koch's publication it may be said that the bacterial causes of only one or two diseases had been rendered probable or certain. I refer particularly to

anthrax and to relapsing fever, in which O. Obermeier (1873) had demonstrated the constant presence of the special organism for long called *Spirochæta Obermeieri*. Between 1881 and 1898 the causes of tuberculosis, glanders, Asiatic cholera, diphtheria, typhoid fever, pyogenic lesions, gonorrhœa, pneumonia, Malta fever, cerebrospinal fever, tetanus, plague, botulism and dysentery were brought to light. Their special history is referred to in other sections of this work. It is remarkable that no important pathogenic micro-organism was discovered by the French School, if we except the trivial communication on *Bacillus pestis*, which apparently was independently discovered by Yersin (1894), and this is the more remarkable when we remember that Pasteur had been one of the pioneers in the early bacteriological period. French bacteriology in the hands of Pasteur and his pupils was developing along other lines, and especially in connection with the problems of prophylaxis and immunity to infective diseases.

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Researches of Pasteur on Attenuation of Virus and Prophylactic Inoculation.

The discovery of the bacterial causes of many infective diseases was made chiefly by German bacteriologists shortly after 1881, when Koch published his method of pure cultivation. In the two previous decades Pasteur had been the chief pioneer, but his line of research was not one that brought him into close contact with disease of man and animals. After 1877 he began, however, to interest himself in such problems, and his early work on anthrax, dating from about this time, has already been referred to. He became interested chiefly in the problems of recovery

from disease, and although nearing his sixtieth year, he suddenly broke new ground in a series of remarkable researches, which exhibited an unexampled fertility in ideas and led to important developments. From the study of the literature on the immunity to smallpox following variolation and Jennerian vaccination, he had an instinct that a similar state of affairs might hold for other diseases, and the first malady that he studied from this point of view was chicken cholera. This disease had been recognized as bacterial by the Italian pathologist Perroncito (1879) and by H. Toussaint (1880) of Lyons. Pasteur obtained growths of the virus in chicken broth, and showed that they were highly infective when inoculated into fowls. He further showed that the virulence of cultures grown in series remains for the most part constant, although there appeared to be conditions in which the virulence was lessened. This enfeeblement of the virus was called by Pasteur (1880[1]) 'attenuation' and he announced the remarkable fact that if fowls are first inoculated with the living attenuated virus of fowl cholera they can withstand a subsequent inoculation of living virus which is lethal for unprotected fowls. He was led to this great discovery by a number of observations. He noted, for example, that the virulence of fowl cholera cultures is not always the same, and that if an animal recovers from the effect of an inoculation the disease is not usually liable to recur. If relapses did occur they were in inverse ratio to the severity of the first attack. He noted that the virulence of a culture depends on the time which elapses between successive cultures (Pasteur, 1880[6]). As the time increases there are signs of progressive attenuation of the virus, as shown by the lessened case mortality, and by the delay in the development of the symptoms. If, for example, the virus is transplanted from medium to medium at intervals varying from days to a month or two, no change is observed in the virulence for fowls. If the interval between two successive inseminations in bouillon is prolonged to 3, 4, 5 or 8 months, the scene changes. Instead of possessing a high lethal effect the cultures become less and less virulent, and the animals, even if they become ill, recover. It was apparently an inspiration which led Pasteur to apply virulent fowl cholera cultures to fowls which had come safely through an inoculation with attenuated cultures. This made him suggest the possibility of prophylaxis by attenuated virus—a principle which he established not only for fowl cholera, but also for anthrax, swine erysipelas and rabies. These researches occupied him exclusively to the end of his active scientific career.

The cause of the attenuation of fowl cholera virus in cultures was believed by Pasteur to reside in the deleterious effect of air and particularly of oxygen. In closed vessels of bouillon, cultures retained their virulence unchanged for periods up to ten months, whereas in vessels plugged with cotton-wool, attenuation took place as above described. Large quantities of attenuated fowl cholera virus were prepared by Pasteur and his assistants, and were inoculated into fowls as a prophylactic against the disease. As a practical proposition the method was not invariably a

success, as there was sometimes a rather high mortality from the prophylactic itself, but the soundness of the principle established by Pasteur was admitted, and he attained great fame by his discovery. The virulence of fowl cholera virus also led him (1888) to recommend the use of living cultures of this micro-organism for the destruction of rabbits in Australia and New Zealand. A preliminary experiment carried out on the estate of Veuve Pommery at Rheims was reported as extraordinarily successful (Pasteur, 1888). From subsequent experiences in the attempted destruction of vermin by other workers it would appear, however, that the method has limitations.

Leaving the subject of fowl cholera, Pasteur returned to the study of anthrax. The extraordinary resistance of anthrax spores had been effectively demonstrated by Koch (1876), and this point was further studied by Pasteur (1879), who found living anthrax spores in the soil, above an anthrax carcass buried ten months previously. New experiments carried out by Pasteur in association with Chamberland and Roux (1880, 1881[2]) led to the view that the spores in a buried anthrax carcass are gradually brought to the surface of the soil by earthworms. Soil, containing earthworms, was artificially infected with anthrax spores, and when the worms were subsequently opened the spores were found in their castings (Pasteur, Chamberland and Roux, 1880). Pasteur (1880[5]) also found anthrax spores in soil, or in worm castings in the part of a field in which three cows had been buried two years previously. A paling was placed round the infected spot and of four sheep made to graze in the enclosure, one died of anthrax. Sheep grazing in a similar but non-infective enclosure only three or four yards away remained well. In soil on a site in which anthrax cadavera had been buried twelve years previously and not afterwards, Pasteur, Chamberland and Roux (1880) demonstrated anthrax spores by inoculation into guinea-pigs.

During these studies Pasteur's attention was directed to the fact that, domestic animals occasionally recover from anthrax, and he began to apply this knowledge to the possibility of preventive inoculation. He found that anthrax bacilli will not grow above 45° C., but that there was a good growth at 42 to 43° C. At the latter temperature the culture became asporogenous, and in a month had died out altogether. When a virulent anthrax culture was kept at 42 to 43° C. for 8 days, it was found to have lost much of its virulence, and was innocuous when injected into guinea-pigs, rabbits or sheep. A longer cultivation at this temperature rendered it still more attenuated in virulence. This was the method which was suggested by Pasteur, Chamberland and Roux (1881[2 & 3]) as an anthrax prophylactic, and to demonstrate its efficiency and establish its importance for agriculture, an experiment was undertaken on a big scale by Pasteur, Chamberland and Roux (1881[5]) at the suggestion of the Agricultural Society of Melun, which placed 60 animals at their disposal. In the yard of the farm Pouilly-le-Fort, a mile from Melun, the experiment took place in the presence of a great concourse of people of all sorts, including the

correspondent of *The Times*, M. de Blowitz, who mailed his 'copy' throughout the world and made the Pouilly-le-Fort experiment famous. The experiment consisted in the inoculation, on May 5th, 1881, of 24 sheep, 1 goat and 6 cows with 5 drops of a living attenuated culture of anthrax bacilli. On May 17th, 1881, all these animals were re-inoculated with a less attenuated culture. On May 31st, 1881, the crucial test was applied. All the 31 inoculated animals received a highly virulent anthrax culture, which was also injected into 24 sheep, 1 goat and 4 cows, not previously inoculated and serving as controls. The result was soon apparent. On June 2nd, 1881, all the vaccinated sheep were well. Twenty-one of the control sheep and the goat were dead from anthrax ; 2 control sheep died in the presence of the spectators, and the last of them died at the end of the day. The six protected cows were well, whereas the four control cows had extensive swellings at the point of inoculation and they also had fever. On June 3rd, one of the protected sheep died. It was pregnant, and an autopsy suggested that it had succumbed owing to the death of the fœtus.

Pasteur's triumph was complete. The discovery of the attenuated anthrax prophylactic constituted, he said, 'un progrès sensible sur le vaccin jennerien'. A few weeks later Pasteur was the chief star at the International Medical Congress in London, and delivered an address on 'vaccination in relation to chicken cholera and splenic fever'. It was in this address that he explained the extended use of the terms 'vaccine' and 'vaccination' to denote the process of prophylactic inoculation in general, and in consideration of the services to mankind of Edward Jenner (Pasteur, 1881).

Pasteur's anthrax vaccine holding out as it did the highest hopes for agriculture soon became general, and by 1882, Pasteur (1882[2]) reported the results on 85,000 animals. In the case of 79,392 sheep the annual mortality from anthrax was believed to have fallen from 9·01 per cent. among the uninoculated to 0·65 per cent. among the inoculated. The vaccine was distributed broadcast and applied in many countries, but not always with the successful results seen at Pouilly-le-Fort. A full account of these early inoculations will be found in the works of Chamberland (1883, 1894). Among the critics of the anthrax inoculation was Koch (1882), who published a reply to an address given by Pasteur (1882[3]) on the 'attenuation of virus' at the International Congress of Hygiene and Demography at Geneva. Koch maintained that on account of the imperfections of Pasteur's method, the unfavourable results, the mortality from the prophylactic itself and the shortness of the immunity produced, anthrax vaccination was not really a practical proposition. Koch was also scathing in his remarks on the purity of the vaccine, and stated that he had found the French product to contain bacteria other than those which should have been present. Pasteur (1883[1]) replied scornfully to Koch's criticism and proceeded quietly with his work, and by 1894 Chamberland reported that over three-and-a-half million sheep had been inoculated against anthrax.

In association with L. Thuillier, Pasteur now studied the disease known in France as ' rouget des porcs ' (angl. Swine erysipelas). Thuillier was sent to Peux (Vienne) and there he discovered a well-defined bacillus in the blood of pigs suffering from the disease. Pasteur himself joined Thuillier at Bollène (Vaucluse) and obtained cultures of the bacillus of rouget (Pasteur and Thuillier, 1882[1 & 2]). Attempts were made to attenuate the virus, but difficulties were experienced owing to the variation in the susceptibility of different races of pigs. The investigations were continued, however, and the remarkable fact was disclosed that the virus of rouget becomes attenuated for the pig by passage through the bodies of rabbits, and this was the source of the vaccine which Pasteur and Thuillier (1883) recommended for inoculation of the pig. The correctness of their conclusions has not been seriously challenged. Between 1886 and 1892 more than 100,000 pigs were inoculated in France, while in Hungary between 1889 and 1894 the number exceeded one million.

Pasteur's work on fowl cholera, anthrax and swine erysipelas aroused world-wide interest and his fame was higher now than ever before. Although crippled physically, his intellect was still active when he entered the last phase of his great career, and for it he chose the study of the inscrutable disease rabies, which for long had had a firm hold on public imagination. Possibly it was this very fact that impelled Pasteur to its investigation, for it cannot be said that rabies was a disease of any great consequence in the general mortality of man or animals.

Pasteur's introduction to rabies was the case of a child in the Hôpital Ste Eugénie in Paris. Four hours after its death, saliva was collected and sent to Pasteur who injected it into rabbits. They died of a disease that was transmissible in series. In the blood of these animals Pasteur found a minute encapsulated microbe in the form of a figure of 8 (Pasteur, Chamberland and Roux, 1881[1]), but from the fact that a similar microbe occurred in normal saliva he concluded that it was not the cause of rabies. The microbe of the saliva was in fact the pneumococcus.

Galtier (1881) showed that rabies is transmissible to rabbits, and this observation no doubt had an influence on Pasteur, who with Chamberland and Roux began a long series of researches on experimental rabies. It was shown that the virus of rabies occurs in the central nervous system. By inoculation of dogs directly under the dura mater Pasteur proved that the lengthy incubation period which follows the subcutaneous inoculation of ordinary rabies virus can be shortened (Pasteur, Chamberland, Roux and Thuillier, 1881, 1882). By passage through the nervous system of rabbits the rabies virus became greatly exalted and the incubation period became shorter. A limit was reached beyond which it was apparently impossible to go. In Pasteur's language the virus had become ' fixe '. This ' virus fixe ' acted with astonishing regularity. As early as 1882 Pasteur and his collaborators had noted the very occasional occurrence of spontaneous recovery in canine rabies, and in one dog that exhibited this phenomenon subsequent intracerebral inoculations made on two

occasions remained without effect. Four other dogs behaved likewise, and it was from these facts that Pasteur attempted to obtain an effective prophylactic against rabies. Large kennels were constructed at Villeneuve l'Etang near St. Cloud, and experiments proceeded. At the International Congress of Medicine in Copenhagen, Pasteur (1884) gave an extensive account of his researches, and announced the results of protective inoculation in dogs. Pasteur (1885) also described a method of protecting dogs *after* they had received the virus from the bite of a rabid dog. The method consisted in suspending in dry, sterile and still air the spinal cords of rabbits which had died after the intradural injection of virus fixe. In the course of about two weeks the cord, at first highly virulent, becomes almost non-virulent. By inoculation of emulsions of the attenuated cord, it was found possible to proceed to emulsions of less attenuated virus, and, finally, ' virus fixe ' could be applied with impunity to the nervous tissue of dogs. These remarkable results on dogs suggested that the method might be applicable to human beings bitten by rabid animals and still in the incubation stage of the malady. Pasteur (1885) had not long to wait for an opportunity to test this suggestion, for a boy, Joseph Meister, aged nine, was brought to him from Alsace on the 6th of July, 1885, suffering from extensive bites inflicted by a dog certainly rabid. Meister was injected with attenuated rabbit spinal marrow which had been kept for 14 days. In a further series of 12 injections he received virus that was stronger and stronger, until on the 16th of July he received an inoculation of virulent spinal marrow only one day after it had been taken from a rabbit, dead from ' virus fixe '. The virulence of the material used in the whole of the 13 injections received by Joseph Meister was controlled on rabbits, and it was shown that in his last two inoculations he had withstood a virus which was of maximal virulence. Meister remained well.

Pasteur (1885) also gave an account of the second case treated. He was Jean Baptiste Jupille, aged 15, a shepherd boy of Villers-Farlay in the Jura. Seeing a suspicious-looking dog about to attack some children Jupille attempted to drive it off with a whip, but was severely bitten. None the less he closed with the brute, wound the thong of his whip round its muzzle, and battered its skull in with his sabot. The dog was subsequently declared rabid, and Jupille was brought to Paris, where six days after he had been bitten his treatment was begun by Pasteur. The result was successful and Jupille's deed was subsequently commemorated in a bronze statue which to-day stands in front of the Pasteur Institute in Paris.

After these early and dramatic successes, numerous patients, bitten by animals that were rabid or presumed to be so, began to arrive in Paris to receive the rabies prophylactic. By October, 1886, no fewer than 2,490 persons had been inoculated, and Pasteur estimated that the failure was only 1 case in 170 treated. Up to 1905, Bernstein (1905) reported that all over the world at least 104,347 persons had received Pasteur's rabies treatment. For his researches on rabies Pasteur received

extraordinary adulation, amounting almost to idolatry, and a movement was set going in France for the erection of a scientific institute where his prophylactic inoculations could be properly given, and where scientific researches on the subjects in which he had so long been a participator could be further developed. The suggestion aroused the liveliest enthusiasm, not only in France but all over the world. Money poured in and the total collected amounted to 2,586,680 francs. The 'Institut Pasteur' was erected in the rue Dutot, Paris, and was inaugurated on the 14th of November, 1888, in the presence of an audience which included the most distinguished persons in France and other countries. Pasteur was there, but his work was done. He was feeble and ill, but still lingered for another seven years, when he died full of honour on September 28, 1895.

The school which Pasteur created gave an enormous impulse to bacteriology, and Pasteur Institutes, based on the model of the Institut Pasteur in Paris, were established in more than 40 places within the next few years. Much of the knowledge we possess to-day has come from such institutes, and the torch which Pasteur set alight still burns vividly after 40 years.

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History of Immunity Doctrines.

The work of R. Koch and L. Pasteur respectively gave two directions to the development of bacteriology. The German school concentrated, at first at any rate, on the isolation and study of individual bacteria, parasitic and saprophytic, and in due course this led to the foundation of

many highly specialized branches, the magnitude of any one of which to-day is such that no individual, however talented and diligent, can hope to cope with it.

For the medical profession pathogenic bacteria have claimed the greatest, indeed, almost the sole interest, but of equal if not greater importance are the saprophytic bacteria, without which continued existence on the earth would be impossible for plants and animals. The French School, under the influence of Pasteur in the last phase of his life-work, concentrated on the problems of the prevention of infective disease by artificial inoculation with virus, and on the nature of the processes involved in the recovery and immunity from disease. The results, profoundly interesting and of great significance, led to the formation of that branch of science now called ' immunology '. By his researches on chicken cholera, swine erysipelas, anthrax and rabies Pasteur directed attention to the practical and theoretical possibilities of immunity. Simple as immunity appeared at first to be, it was not long before it was found to be a very complicated and variable process. Natural immunity was differentiated from acquired immunity, and by the end of the eighties of last century, the new immunity science had begun to develop in two directions. On the one hand the Pasteur method of prophylaxis by attenuated virus was further advanced along his lines, and on the other, theories were formulated to explain the nature of the immunity process in general. With regard to the practice of inoculation a forward step was taken by D. E. Salmon and Theobald Smith (1884–86), who showed that dead virus can induce an immunity against living virus. Their experiments were carried out on pigeons inoculated with the so-called hog-cholera bacillus. After injection with bacilli killed by heat they found that animals could withstand with impunity doses of living hog-cholera bacilli fatal to unprotected pigeons. This discovery became the basis of all the methods employed subsequently on a gigantic scale to protect human beings against cholera, plague and typhoid fever. The names of W. Haffkine, A. E. Wright and R. Pfeiffer and Kolle were associated with this advance, and A. E. Wright subsequently developed the idea of therapeutic as opposed to prophylactic inoculation along the same lines.

At an early stage investigators occupied themselves with the mechanism of immunity, and two hypotheses developed side by side. The one explained immunity as due to the occurrence of substances in the blood-stream and fluids of the body. These substances were inimical in some way to the development of the infective agent, and for this reason they were spoken of as antibacterial, protective or defensive. This was the humoral theory of immunity. The other view, formulated by E. Metchnikoff, attributed the immunity to a cellular rather than a humoral activity, and for a decade or more (1880–90) the question of humoral versus cellular immunity was fought out on a big scale.

That the fluids of the living body, and especially the blood, possesses some property of inhibiting putrefaction was indicated by Timothy Lewis

and D. Cunningham in 1872, and more exactly by M. Traube and Gscheidlen (1874). The latter showed that arterial blood, drawn aseptically from rabbits, 24 to 28 hours after they had been inoculated with bacteria-containing fluids, did not become putrid, but that this anti-putrefactive property had limits. These observations were confirmed by Watson Cheyne (1879) and Grohmann (1884), and were made the subject of careful study by J. Fodor (1886), a Hungarian, who emphasized the protective or defensive significance of the process. His work was repeated by Wyssokowitsch (1886), who showed that bacteria injected into the blood-stream disappeared therefrom, but did not leave the body by any of the channels of secretion or excretion. He was opposed to Fodor's idea of destruction, and inferred that the injected bacteria really became deposited in the internal organs, such as the spleen, liver and bone-marrow. Fodor (1887, 1890) showed, however, that there was evidence of extravascular destruction, for when he injected blood *in vitro* with anthrax bacilli and kept it at 38° C., a rapid diminution of bacteria was found to have taken place, as judged by enumeration of colonies in plate cultures. It was conceivable that, as the blood had undergone clotting, the bacteria might have become entangled in the coagulum, the result being a fallacious appearance of actual destruction. This criticism was set aside by G. H. F. Nuttall (1888) who employed defibrinated blood. Working in Flügge's Institute in Göttingen, he took blood from a vessel and defibrinated it with sterile quartz. Known quantities of bacteria were now added to the blood *in vitro*, and plate cultures were made at intervals. Nuttall tested the blood of rabbits, mice, sheep, pigeons and dogs on anthrax bacilli, and he clearly showed in a number of instances that there was a profound diminution in the number of anthrax bacilli. His results were often striking. Thus a suspension which in four controls yielded an average of 15,730 anthrax colonies showed no colonies or only five colonies one hour after exposure to defibrinated blood. Nuttall also found that the bactericidal power of the blood disappeared after a time, and, in the case of dog's blood was destroyed when the blood was heated to 52° C. for 10 to 30 minutes. Rabbit's blood was rendered inert if heated to 55° C. for 45 minutes, although it was still active at a temperature of 48 to 50° C. for 10 minutes.

The essential facts disclosed by Nuttall were confirmed by many workers, and especially by F. Nissen (1889) and by Hans Buchner (1889, 1890) alone and in association with various co-workers (1890). Nissen found that the bactericidal power of blood varies according to the bacteria employed in the test, some being killed and others not. Behring and Nissen (1890) found that, whereas the serum of normal guinea-pigs is incapable of killing *Vibrio metchnikovi*, the serum of these animals, immunized against this organism, exerts a marked bactericidal action. This was not, however, of general applicability, as it did not occur in the case of animals immunized against pneumococcus. About this time (1890) Hans Buchner in Munich began a long series of publications on the

nature of immunity and bactericidal action. He showed that bactericidal substances occur not only in the blood, but in the cell-free serum, not only *in vivo* but also *in vitro*. He named these substances 'alexines' (ἀλεξειν to defend) from their defensive or protective properties. This 'alexic' theory held the ground for a number of years despite the immediate criticism of Metchnikoff (1890), Jetter (1891–2), and others, but ultimately was found to be untenable, at any rate in the simple form proposed by Buchner.

While the humoral theory was being developed the idea that an active part is played by the infected host in natural immunity was advocated by the Russian zoologist, Metchnikoff, and was developed by him in a long series of researches. The central idea of his teaching was that infective bacteria introduced into the body are destroyed by the agency of certain cells, and he pictured the destruction as the result of a process of intracellular digestion. The cells which possessed this property were called by Metchnikoff (1884[1]) 'phagocytes' and the process, 'phagocytosis'.

That cells can engulf foreign matter was of course known to microscopists long before Metchnikoff's time. Amœbæ and other protista obtain their nourishment in this way. Langhans (1870) had shown that, in hæmorrhagic foci, leucocytes become charged with the debris of red blood corpuscles, and Birch-Hirschfeld (1872) had found that cocci injected into the blood-stream may be taken up by leucocytes. Panum (1874) had indeed suggested that this process might actually be a method of destruction of microbes—an opinion also shared by K. Roser (1881). R. Koch (1876) had described and figured anthrax bacilli inside cells in the dorsal lymph sac of the frog after particles of anthrax spleen had been implanted in that site (Fig. 7 on Fig. 21), and he had seen intracellular incorporation of anthrax bacilli in the spleen itself of a horse dead from anthrax. These observations were not appreciated or were overlooked, when Metchnikoff about 1883, working at Messina, made observations on starfish and other animals, and concluded from what he saw that certain mesodermic cells possess real digestive powers. He extended his idea of intracellular digestion in a number of directions, and began to apply it as an explanation of immunity. This aspect was first studied by Metchnikoff (1884[1]) in the case of *Daphnia magna* infected by a parasite, *Monospora bicuspidata*. He, later, extended his idea to bacterial infections such as anthrax (1884[2]) and erysipelas (1887). A wide dissemination of Metchnikoff's views took place through the publication of his remarkable 'Leçons sur la pathologie comparée de l'inflammation' (1892) and in various articles dealing with immunity.

The phagocytic theory was adversely criticized by P. Baumgarten (1889), by Weigert (1891) and others, and for a time the humoral doctrine of immunity seemed to be in the ascendant. It was suggested, however, that the two theories might be brought into line on the supposition that bactericidal substances in the serum are really cell products. An attempt of this kind by Hankin with his theory of alexocytes dates from about

1890. A great mass of material continued to be published on both theories, but it gradually emerged that neither the one nor the other was capable of explaining all the facts of immunity. Before dealing with the further development of the question it is necessary to refer to the progress of the humoral doctrine which was brought about by the discovery of antitoxin by Behring and Kitasato (1890).

It was in connection with the immunity to tetanus and diphtheria that this discovery was made. Behring and Kitasato published their first account on December 4, 1890, in a brief but epoch-making paper dealing with the artificial production of immunity to tetanus. It contained the remarkable statement that the immunity of rabbits and mice which have been treated with tetanus cultures depends on the capacity of the cell-free blood-serum to render innocuous the toxic substances elaborated by the tetanus bacillus. This capacity was so durable that it still exhibited its activity when the immune serum was injected into the bodies of other animals. By means of immune serum Behring and Kitasato were able to protect mice which received 300 minimal lethal doses of tetanic poison. Normal serum had no such property. In a footnote in their paper the toxin-destroying property of the immune serum was described as ' antitoxic ', and this word was employed everywhere at once and has persisted. One week after the joint publication of Behring and Kitasato on tetanus immunity, Behring alone published a paper (December 11, 1890) on immunization against diphtheria, and he confirmed for this infection all the essential facts which in conjunction with Kitasato he had demonstrated for tetanus.

The possibility of serum therapy was opened up by these investigations. Priority claims have been made by, or on behalf of, Héricourt and Richet (1888) and Babes and Lepp (1889), but history must assign unchallenged to Behring and Kitasato the discovery of antitoxic immunity. In rapid succession appeared a large number of papers confirming the existence of antitoxin in the serum of animals immunized against tetanus and diphtheria, and extending our knowledge of the antitoxic process. Prominent among the workers may be mentioned Behring and Wernicke (1892), Brieger and Cohn (1893), Behring, Boer and Kossel (1893), Aronson (1893), Behring and Ehrlich (1894), and Ehrlich, Kossel and Wassermann (1894). The first person treated by diphtheria antitoxin was a child in von Bergmann's Clinic in Berlin, the injection being made by Geissler on Christmas night, 1891 (Wernicke, 1913). A remarkable impulse was given to the use of diphtheria antitoxin in man by the work of Roux and L. Martin (1894) in Paris, their results being communicated by Roux (1894) in an address at the International Congress of Hygiene and Demography in Budapest in that year.

From the scientific point of view great credit must be given to Paul Ehrlich (1897[2], 1898) who in most laborious researches established the principles for the standardization of bacterial toxins and antitoxins and laid down the technique employed to-day. His ' Werthbemessung

des Diphtherieheilserums und deren theoretische Grundlagen ' (1897[2]) is a classic in the bacteriological literature. The experiments for this great work were carried out chiefly in two small one-storey houses in Steglitz (Berlin). Later (October, 1899), he transferred his activities to a large institute—the ' Kgl. Institut für experimentelle Therapie ' in Frankfort-a-M, and to this was subsequently added (1906) the Georg-Speyer-Haus founded by Frau Franziska Speyer.

Following the discovery of Behring and Kitasato it was soon found that the ability of the animal organism to produce antitoxin is a widely spread phenomenon, and results from the injection of many substances classed as poisons, of animal as well as of vegetable origin. Of historic importance were the researches of Ehrlich (1891) on ricin and abrin. He showed that each of these vegetable poisons produces an antitoxin which is specific. He it was, also, who pointed out the important differences between active and passive immunization (Ehrlich, 1892). The success attained in the treatment of diphtheria by specific antitoxic serum early led to the hope that similar specific antitoxins might be prepared from other infective agents such as those producing pneumonia, typhoid fever, cholera, anthrax, tuberculosis and suppuration. It soon became clear, however, that the serum of animals inoculated with such agents or their products is not similar in action to diphtheria antitoxin. In some instances, indeed, the ' immune ' serum apparently had no properties which could not be found in normal serum. In other cases, as, for example, in typhoid and cholera, the immune serum had definite, specific properties, but exhibited them in a manner different from that of antitoxic sera.

It was in connection with cholera immunity that R. Pfeiffer (1894) began his classical researches, which showed that a destruction of cholera vibrios takes place by a process which he called ' bacteriolysis ' (Pfeiffer and Issaeff, 1894). Cholera vibrios were found to be destroyed *in vitro* in the serum of animals immunized against cholera vibrios, and the same effect took place in the peritoneal cavity of the living immunized animal. On removing drops of peritoneal fluid by means of a capillary pipette, Pfeiffer found that in the case of the immune animal, the injected cholera vibrios were almost instantly rendered motionless and swollen. They then changed into micrococcus-like bodies (' spherulation ') which became more and more difficult to see, until finally they vanished from view altogether. This process of dissolution or ' lysis ' occupied about twenty minutes, and was apparently quite independent of any cellular intervention. Pfeiffer showed also that the cholera serum acts *in vitro*, but its activity is abolished by dilution or by heating at 60° C. The solution or lysis of bacteria in the peritoneal cavity of an immunized animal is spoken of as ' Pfeiffer's phenomenon ', and is, as Pfeiffer showed, highly specific, in that vibrios closely related to *Vibrio choleræ* are not destroyed by cholera serum. He also observed that cholera immunity could be transferred passively, for when anticholera serum from an immunized guinea-pig was injected into the peritoneal cavity of a normal guinea-pig cholera

vibrios subsequently introduced into the latter underwent the characteristic bacteriolysis. C. Fraenkel and Sobernheim (1894) found that cholera-immune serum, even when heated to 70° C. for 1 hour, can protect normal guinea-pigs against lethal doses of living cholera vibrios, and in this case likewise the vibrios are destroyed by the lytic process in the peritoneal cavity. As such heated serum is inert *in vitro* and it apparently becomes active *in vivo*, R. Pfeiffer regarded the active agent as a kind of proenzyme which became converted into an enzyme in the living body.

Although the observations reported by R. Pfeiffer were at once confirmed, some alternative interpretations were made as a result of added knowledge. Thus Metchnikoff (1895) showed that the Pfeiffer phenomenon takes place *in vitro* if the precaution is taken of adding some normal peritoneal exudate to the mixture of cholera vibrios and heated cholera serum, and in the same year J. Bordet (1895) showed that normal blood-serum can be used as a substitute for normal peritoneal exudate. Bordet also confirmed Pfeiffer's observation that cholera-serum acts specifically, for on mixing it with vibrios other than *V. choleræ* no lysis took place. It was apparent, therefore, in experiments on the bacteriolysis of cholera vibrios that heated immune serum cannot produce this effect, but that a mixture of unheated normal serum and heated immune serum can. On heating normal serum to 55° C., Bordet found it incapable of rendering heated immune serum active, and he concluded that two factors or agents must be concerned in producing bacteriolysis. One of the substances is present both in normal and in fresh immune serum and is thermolabile, the other is peculiar to the immune serum and is thermostable.

These classical experiments of Bordet were at once confirmed everywhere, and a lytic action was found to be applicable to cells other than bacteria. Thus red blood corpuscles were found to be destroyed by a similar process—hæmolysis, and this discovery was in the main made by Bordet. Hæmolysis was subsequently studied in the most minute way, and was found to reveal facts of great importance for the immunity doctrines. It was long known from observations by Creite (1869) and Landois (1875) that the serum of certain animals is destructive to the blood corpuscles of other animals, but no great interest was evinced in the fact. Daremberg (1891) and Hans Buchner (1893[1]) also noted that a serum which is hæmolytic ceases to be so if heated to 55° C. In 1898 Belfanti and Carbone showed that the blood-serum of horses which had been injected with the red blood corpuscles of rabbits, acquired toxic properties for the latter animals. Bordet (1898) recognized the significance of this discovery, found that it was a general principle and showed that in its mode of action hæmolysis behaves like bacteriolysis. Bordet's paper on the subject opened up a new field of enquiry which was soon enlarged by many workers, the most prominent among whom were Ehrlich and Morgenroth (1899; 1900).

The essential facts established by Bordet (1898) were that when rabbit's blood was injected into guinea-pigs, the serum of the latter acquired the property of dissolving the blood corpuscles of the former *in vitro*. On adding to a suspension of rabbit's blood corpuscles in salt solution a small quantity of guinea-pig immune serum the blood corpuscles of the rabbit clumped together (agglutination), and the hæmoglobin passed into the medium leaving behind the colourless stromata or ' shadows '. Heated to 55° C. for half an hour the hæmolytic property of the immune serum was found to have disappeared. If then to a mixture of rabbit's blood corpuscles and inactive (i.e. heated) guinea-pig immune serum Bordet added a quantity of serum from a normal guinea-pig or a normal rabbit, the phenomenon of hæmolysis soon showed itself. The guinea-pig immune serum was found to have no effect on the red blood corpuscles of guinea-pigs or pigeons. In fact it had a specific effect only upon the corpuscles of the animal (rabbit) with which the guinea-pig had originally been injected. Bordet also showed that a hæmolytic destruction of corpuscles took place in the peritoneal cavity of an immunized guinea-pig. If the immune serum was heated to 55° C. and was then mixed with the homologous red blood corpuscles, the latter were hæmolysed in the peritoneum of a normal guinea-pig.

Taking up the subject of hæmolysis at this stage, Ehrlich and Morgenroth (1899; 1900) confirmed and extended the fundamental work of Bordet. In the nomenclature of Bordet the thermolabile element in hæmolysis was named ' alexine ' and the thermostable element the ' substance sensibilisatrice ', whereas Ehrlich and Morgenroth called them ' complement ' and ' amboceptor ' respectively—names which are almost universally used to-day. The further development of the hæmolytic question is outside the scope of the present work and will not be considered except in its applications to bacterial diagnosis.

Even before the subject of hæmolysis had been worked out, it was noted that immune serum may exhibit properties which are neither antitoxic nor lytic. As far back as 1889 Charrin and Roger found that when a culture of *B. pyocyaneus* was grown in the fluid serum of an animal which had been injected with that organism, it did not grow diffusely, as it does in broth or in normal serum, but in small masses which sank to the bottom of the tubes. On examination of these masses the bacilli were found to have become stuck together. Similar appearances were noted by Metchnikoff (1891) in the case of *Vibrio metchnikovi* and pneumococci grown in their respective immune sera, and he pointed out the importance of the change and the need for its further study. The clumping of bacteria is now called agglutination, and, since these early observations, has been the subject of an extraordinary amount of study. The first systematic investigation of the agglutination process was published by Herbert Durham, who communicated results of experiments which he had carried out with Max Gruber in the Hygiene Laboratory in Vienna and by himself in Guy's Hospital, London. Durham's paper was read in abstract

at a meeting of the Royal Society on January 3, 1896, and this was followed by communications by Gruber (March 3, 1896) and by Gruber and Durham (March 31, 1896).

Even in Durham's first paper attention was directed to the possibility of the agglutination method as an aid to the diagnosis of disease, and this application was actually made by F. Widal (June 26, 1896) and by A. S. Grünbaum (September 19, 1896) in the case of enteric fever. Known for many years as 'Widal's test' or 'Gruber-Durham reaction', it is nowadays spoken of simply as the 'agglutination reaction'. This reaction is brought about by the addition of a specific immune serum to a homogeneous suspension of bacteria. From the first the reaction aroused great interest and was practised everywhere in innumerable technical forms as a diagnostic test for typhoid fever, and as a standard test for the differentiation of allied bacteria. The literature on the subject is one of great dimensions and cannot be followed in detail here. The two aspects of the agglutination problem which have received the greatest attention are its specificity and its nature. With regard to the former, the existence of 'group agglutination', i.e. a common agglutinating reaction acting on a group of bacteria closely allied to each other, was emphasized by Pfaundler (1899) and by H. E. Durham (1900–1).

Bordet (1899), in his classical paper on the mechanism of agglutination, drew attention to the remarkable fact that agglutinins are absorbed from serum when the latter is saturated with the homologous micro-organism. He showed that if normal horse-serum was mixed with cholera vibrios, and, after contact, the vibrios were removed by means of centrifugalization, the clear supernatant fluid no longer retained its agglutinating property for cholera vibrios, whereas its agglutinating power on typhoid bacilli persisted. Conversely, by saturating serum first with typhoid bacilli the typhoid agglutinins were removed, whereas the supernatant liquid still agglutinated cholera vibrios. This principle was utilized by A. Castellani (1902) with his absorption of agglutinin test, which is extensively employed at the present day. With regard to the mechanism of agglutination, the experiments of Bordet (1899) and A. Joos (1901, 1902) were of great importance, but the subsequent development of the problem cannot be dealt with here. It may merely be stated that work on the subject is still in progress.

During the time that the facts respecting agglutination were being brought to light, R. Kraus (1897) discovered still another property of immune serum. By injecting into animals the clear filtrates from liquid cultures of cholera, typhoid or plague bacilli, he obtained sera which produced precipitates when mixed *in vitro* with samples of the filtrates with which the animals had been injected. Thus the serum of animals injected with cholera culture filtrates precipitated the latter, but did not cause a precipitate in the filtrates of other cultures. Kraus's discovery of precipitating sera ('precipitin') was confirmed by many workers, and Bordet (1899) found that it also occurs with non-bacterial substances.

He showed, for example, that cow's milk, injected into rabbits, gave rise to a serum (' lactoserum ') which was capable of producing a visible precipitate when added to milk. Tchistovitch (1899) showed that the same phenomenon of precipitation occurs in the case of eel-serum and its homologous antiserum. Since that time precipitins have been prepared from an enormous number of protein substances of animal or vegetable origin, and their specific characters have been utilized in medicine and in industry for the differentiation of substances otherwise indistinguishable.

In the period 1888–1900 a large number of facts had been accumulated on the subject of immunity, not only against bacteria, but also against many chemical substances of protein constitution. It became evident that after parenteric injection of such substances the animal body develops others which act in a specific manner contrary to the substance injected. These contrary substances appear in the blood-serum and other body fluids, and have collectively been called ' antibodies '. The substances which evoke antibodies have been called ' antigens '. One of the most remarkable properties of antibodies is their specificity—a fact which was clearly enunciated by Behring and Kitasato (1890) in their work on antitoxin. This specificity early attracted the notice of others, and gave rise to the idea that the antitoxin must be produced from the toxin (antigen). Thus, Hans Buchner (1893[2]) considered that antitoxin was merely a non-poisonous modification of toxin. The more, however, that the problem was studied, the less likely appeared this view, which in fact was rendered highly improbable by the experiments of Roux and Vaillard (1893) and of Salomonsen and Madsen (1898), which made it clear that antitoxin in the serum was something created in the body as a result of the injection of toxin. The antibody was found not to appear immediately after the injection of toxin. There was a latent period, which was first noted by Ehrlich (1891) in the case of ricin antitoxin. On feeding mice with ricin he found that for the four following days they showed no increased resistance to ricin and very little on the fifth day. By the sixth day, however, the state of immunity had been critically developed, and was accompanied by the presence of antitoxin in the blood. This course of antitoxin formation has since been found to be applicable to other antibodies. After the critical entry of the antibody in the serum, fluctuations occur, the course of which was studied by Brieger and Ehrlich (1893), Salomonsen and Madsen (1898), Morgenroth (1899), and others.

The certain fact that the animal body generates antibodies early led to attempts to locate the site of formation, but in spite of many investigations pursued down to recent years it cannot be said that the problem is yet solved. The transmission of immunity from parents to offspring was also extensively studied, especially by Ehrlich (1892).

The general result of a large amount of experimental work carried on for many years was clearly to show that the injection of an antigen is followed by the appearance in the humours of antibodies. The antibodies

vary in type, but each is specific for its antigen. The antibodies are formed by the animal, run a well-defined undulatory course, and are associated with a corresponding state of immunity. This immunity is not transmitted according to the general laws of inheritance.

This is a brief statement of the facts which emerged from many experiments. The innate tendency of the human mind for the concrete rather than the abstract early led to attempts to formulate explanations as to how these things are brought about. In this way arose various theories of immunity, some of which have already been referred to. Pasteur (1880) vaguely stated what was called the ' exhaustion ' theory, according to which immunity was due to the disappearance from the body of some necessary foodstuff, which was used up during the first attack of the micro-organisms. This view was rendered very improbable by the experiments of Bitter (1888) and is also improbable on theoretical grounds. Chauveau's ' retention hypothesis ' (1888), according to which the microbe in its first invasion left behind some antiseptic-like substance, was not found to be in complete accordance with the facts. Both these hypotheses failed to realize the cardinal importance of active interference by the tissues of the infected animal, and this was first clearly enunciated by Metchnikoff in his ' phagocytic ' theory, reference to which has already been made. It was while Metchnikoff was collecting data in support of his phagocytic hypothesis that the humoral or alexic theory of Buchner began to develop. These hypotheses, at first markedly antagonistic, were brought into line with each other, especially through the researches of Denys and his collaborators in Louvain. In particular the experiments of Denys and Havet (1894) caused Buchner to amend his views. The Belgian workers found that dog's serum is less bactericidal than dog's blood as a whole. Working with *B. coli*, *B. subtilis* and *Staphylococcus* they found that the leucocytes play a fundamental role, for, when they were removed, the plasma was found to have been deprived of a considerable part of its bactericidal powers. Denys and Havet considered that the leucocytes act conjointly with the blood fluids in a manner varying with the animal species, and also probably with the micro-organism examined. This view of conjoint action was supported later by the experiments of A. E. Wright and Douglas (1903) in their ' opsonic ' theory. By the simple expedient of testing separately and in conjunction the serum and leucocytes, they showed that substances—' opsonins '—(ὠψωνέω, I cater for) exist in the serum, and that their action is in some way to alter the microbe so that it falls an easy prey to the leucocytes. The experiments of Wright and Douglas aroused a good deal of interest, but their interpretation of the nature of the bodies concerned was adversely criticized by several workers.

By the end of the nineteenth century it had become apparent that the whole problem of immunity was one of great complexity. It was known that in some way or other antibodies neutralize the effects of the homologous antigens, but the manner in which this takes place was the

subject of much discussion, and may be said to be not yet completely cleared up. A great deal of the answer to the question concerns the neutralization of toxin by antitoxin, and the earliest view was that the toxin was simply destroyed by the antitoxin. Hans Buchner (1893) opposed this view when he found that a tetanus toxin-antitoxin mixture neutral (i.e. without effect) on mice was nevertheless lethal for guinea-pigs.

Calmette (1895) working with a neutral mixture of snake venom and antivenom showed that by heating the mixture to 68° C. it became as toxic as if no antivenom had been present. The subject of the interaction of toxin and antitoxin was extensively studied by Ehrlich, whose work was of fundamental importance. It is well known that solutions of ricin produce in extravascular blood an agglutination of the red blood corpuscles. This agglutinative reaction can be inhibited by antiricin. On studying this relationship, Ehrlich (1897[1]) found that the same dose of antiricin which *in vitro* could not prevent agglutination was also unable *in vivo* to prevent a fatal ricin intoxication. On the other hand, a quantity of antiricin which *in vitro* prevented the agglutinating action of ricin on the red blood corpuscles also completely protected mice inoculated with lethal doses of ricin. Finding that there was apparently a direct union of ricin and antiricin *in vitro*, Ehrlich concluded that it was a chemical process, and in all his numerous subsequent publications he maintained this view. Following the law of constant proportions, he considered that a definite quantity of a toxin united with a constant unalterable quantity of the homologous antitoxin. Ehrlich's experiments on ricin-antiricin were repeated, and others were added by many workers in the subsequent years. The results seemed to show that the antibody acts directly on the antigen.

The next problem to be determined was the fate of the toxin in a toxin-antitoxin mixture. The observations of Calmette on venom-antivenom have been referred to. Important additions to knowledge were made by Ehrlich, who was the first to emphasize the necessity of exact quantitative measurements in dealing with the question. He showed that the union of toxin and antitoxin was accelerated by heat and retarded by cold. The reaction was found to proceed more quickly in concentrated than in dilute solutions. Such facts indicated analogy with chemical processes, and Ehrlich believed that the substances concerned would react in equivalent proportions. He was quickly disillusionized by finding that when different diphtheria toxins were balanced against one and the same diphtheria antitoxin, the quantitative relations were quite different. He now began an extremely elaborate analysis of the whole question, and the results which he obtained have formed the basis of the methods of standardizing antitoxin. In the course of his work he elaborated a complicated hypothesis which exerted a profound influence on the development of the whole problem of immunity, and surrounded itself with a vast mass of literature.

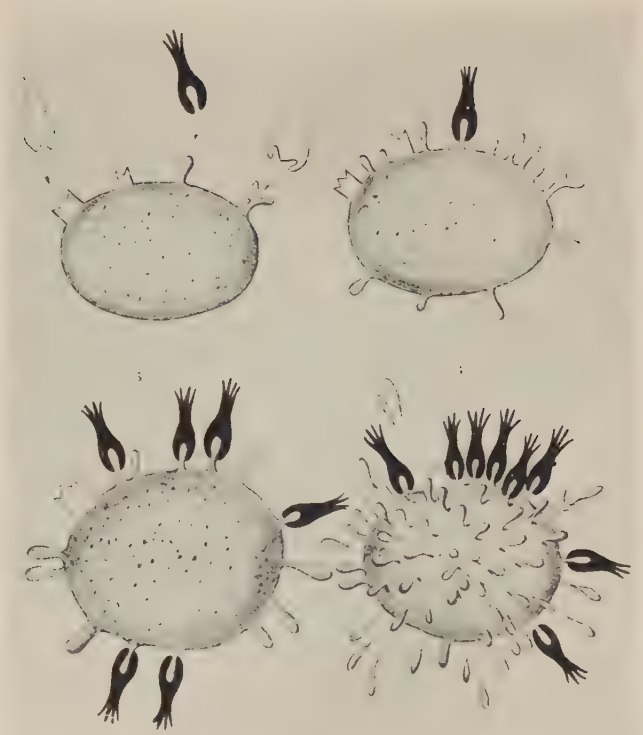
One of the fundamental ideas which Ehrlich was led to hold was that the lethal action of a toxin and its antitoxin-combining power are two separate functions. He explained this by assuming that the toxin contained two different atom groups, one of which, the ' haptophore ', (ἅπτω, I seize), conditions the union with the antitoxin, whereas the other—the ' toxophore '—is, as its name implies, the carrier of the toxic action. Of the two, the haptophore was found to be the more stable. Ehrlich considered that the toxophoric atom group can deteriorate into a non-toxic state—toxoid—although the haptophoric group at the same time remains unaltered. These ideas were not mere whims, but resulted from the experiments which Ehrlich made on a very large scale. Starting with ideas of this kind, Ehrlich carried out most exhaustive researches on the constitution of a number of different samples of diphtheria toxin, and in addition he (1897[2]) put forward a general theory of immunity, which, under the name of the ' side chain ' or ' receptor ' hypothesis, had for many years an enormous vogue, and permeated many branches of scientific medicine and biology. Ehrlich's view on the toxophoric and haptophoric components in a toxin have already been referred to, likewise his belief that a definite quantity of toxin or its toxoid equivalent combined with a definite quantity of antitoxin. There was also a sound experimental basis for the belief that antitoxin is generated in the body as a result of the injection of toxin. From these facts Ehrlich then conceived the idea that a poisoning by a bacterial or other toxin must be due to a specific union of the cells, of some part of the animal economy, with the haptophoric complex of the toxin. In his judgment, it was this very union of specific complexes which gave birth to what is called antitoxin in the serum. Following the conceptions of organic chemistry, Ehrlich pictured the union as one of atom groups—side chains—and he was supported by ideas which he had developed years before on the chemical structure of protoplasm. In his remarkable book, ' On the oxygen requirements of the organism ', Ehrlich (1885) had visualized protoplasm as consisting of a giant molecule equipped with a large number of side chains, the function of which was the fixation of food-stuffs for the nutrition of the cell. He considered that there must be a special executive centre (' Leistungskern ') with attached side chains of subordinate importance as far as concerned the actual living character of the protoplasm. Returning to this conception, Ehrlich (1897[2]) now supposed that it explained antitoxin formation. When the side chains of particular cells have been brought under the influence of a toxin through the intermediation of the haptophoric complex of the latter, the cell was thereby rendered physiologically defective. In order to compensate for this defect, Ehrlich supposed that the cell replied with regeneration of like side chains, and he supported himself in this view on a general biological principle formulated by C. Weigert (1896) of an excessive cell regeneration beyond the actual demand. Weigert's idea was a morphological one, but Ehrlich took it in order to explain the

excessive regeneration of side chains which he had to assume in order to account for antitoxin formation. In Ehrlich's view an excess of specific side chains was produced, and, being ultimately cast off into the blood-stream, really constituted antitoxin. In other words antitoxin, according to Ehrlich, was the cast off receptors or side chains of certain cells for which the haptophoric complexes of a particular toxin have a specific affinity.

This famous theory, partly chemical, partly biological, gave rise to an enormous amount of discussion, and many of its consequences were carefully investigated. A full account of it was given by Ehrlich (1900) in his Croonian lecture before the Royal Society, and it was chiefly in this lecture that he introduced extensions to explain the action and mode of formation of lysins and other antibodies. He regarded the immune-body element of a lysin as essentially an amboceptor combining with the cell on the one hand and with the alexine or complement on the other (Figs. 28 and 29). This idea of an amboceptor was taken up by most workers and has persisted until to-day, notwithstanding that it was doubted by Bordet from the very first. Ehrlich ultimately pictured receptors as belonging to three distinct orders, corresponding respectively to antitoxins, to agglutinins and precipitins, and to lysins.

Although it may be said that Ehrlich's theory met in general with acceptance, there were some critics who raised their voices in opposition. Gruber and v. Pirquet (1903), indeed, went so far as to assert that the whole fabric raised by Ehrlich was in its essence erroneous and not in accordance with facts. Forssman (1908) also published work which attempted to undermine the foundations of the theory, but in general it may be said that it survived the shock. The original interpretation placed by Ehrlich on his quantitative experiments on toxin-antitoxin union remained the standard one, but new ideas were introduced by Danysz (1902) and Arrhenius and Madsen (1902). Thus Danysz discovered a peculiar ' effect ' since known by his name. In connection with ricin and antiricin mixtures, he showed that a mixture of *a* parts of ricin with *b* parts of antiricin was less toxic when the two components were mixed simultaneously than when one-half of *a* was added to *b*, and the other half of *a* was added 24 hours later. This fact, which was confirmed by other workers, was considered to be adverse to Ehrlich's idea of a chemical union in fixed proportions. Arrhenius and Madsen (1902) also came to the conclusion that the neutralization of toxin and antitoxin did not proceed, as maintained by Ehrlich, in step-like formation, but was gradual and the curve was similar to that in which one weak acid was neutralized by one weak base, such as boric acid and ammonia.

Long discussions followed, supporting now one side and now the other, and by degrees a new view of the mechanism of toxin-antitoxin reaction began to make its appearance in the literature. It was considered that the union of the antigen and the antibody was not really a chemical process, as Ehrlich and Arrhenius had supposed, but is akin to what had been called ' adsorption '. Landsteiner and Jagić (1903) and Bordet (1903)



FIGS. 28 and 29.—Ehrlich's figures representing his side-chain hypothesis. From Ehrlich (1900).

[*To face p.* 98.

were early upholders of this view, which has gradually gained wide acceptance, and has led to the production of an extensive literature—still growing. New theories of immunity have ceased for the present to be formulated, and although modern workers seem inclined to jettison Ehrlich's theory they offer no substitute.

Notwithstanding, immunology as a science continues to grow and has far outstripped its original bacteriological limits. The subject has now reached dimensions with which it is impossible to deal and which do not yet come within the scope of history. A word, however, may be said on ' anaphylaxis ', which is almost a branch of immunology by itself to-day. The original data on which the idea of anaphylaxis is based we owe to Portier and C. Richet (1902), who discovered the remarkable fact that certain poisons of animal origin evoke a condition of hypersusceptibility instead of immunity, and that, as a result of this increased sensitivity, death may ensue from a dose of the poison which would otherwise be ineffective. By maceration of the tentacles of certain sea anemones, Portier and Richet obtained a poisonous fluid which, injected intravenously into dogs, in a dose of 0·2 gm. per kgm., produce hypothermia, diarrhœa and death in two to three days. Doses less than 0·2 gm. produced only transient symptoms. If, however, several weeks after the injection of such a dose a second sublethal dose was administered, violent symptoms of poisoning set in and might cause the death of the animal even in an hour, but mostly in 12 to 24 hours. The condition was spoken of—perhaps unfortunately—as anaphylaxis, which was intended to convey the idea of the opposite of protection. These remarkable results were extended in a number of papers by C. Richet alone, and he it was, who showed that this experimentally produced hypersusceptibility was really a widespread phenomenon. He recognized that all the poisons which could produce the anaphylactic effect are of protein character, and that as a result of the first injection something was produced which, in combination with the re-injected poison, gives rise to a new toxic substance—apotoxin—which is the agent causing the anaphylactic symptoms. Since the researches of Richet, an enormous mass has been published on anaphylaxis, but the subject is by no means exhausted yet.

The whole field of immunity continues to be explored and opinions and results are often contradictory. The years of labour have, however, not been in vain, for important practical results have accrued both with regard to the diagnosis and treatment of disease in man and animals, to mention one aspect alone. A great field has been opened up under the name of sero-diagnosis, and has had many applications. The basis on which the value of this method depends is the remarkable specificity which attends immunity reactions. It is true that the specificity is not absolute, but varies qualitatively and quantitatively within certain limits. By means of immune serum search may be made for an unknown antigen and vice versa. The possibility of such a practical test was first suggested by R. Pfeiffer (1894), who found that the bacteriolytic destruction of cholera

vibrios in the peritoneum was so specific that the method could be utilized for the differentiation of cholera vibrios from vibrios otherwise indistinguishable. Bordet (1895) utilized this principle *in vitro* instead of *in vivo*. A great extension of the sero-diagnostic method took place following the discovery of agglutination, and Durham (1896) early foreshadowed its practical application for the differentiation of allied bacteria. Widal (1896) went a step further by employing known bacteria (e.g. typhoid bacillus) to determine the presence or absence of specific agglutination in the serum of the patient presumed to be suffering from enteric fever. Previously unknown fevers inside the enteric group were discovered by sero-diagnosis. It was by this means that Schottmüller (1900) discovered paratyphoid fever. The application of specific precipitating sera in forensic medicine, in hygiene and in the industries is thoroughly established and routine. Of particular medical importance is the so-called complement-fixation test, the principle of which was discovered by Bordet and Gengou (1901). If to a quantity of unheated normal guinea-pig serum was added a quantity of sensitized red blood corpuscles (i.e. corpuscles charged with specific amboceptor), hæmolysis occurred. Bordet and Gengou, however, made the remarkable observation that if cholera vibrios were added to such a mixture they remained intact instead of undergoing spherulation and lysis. The same occurred if the cholera vibrios and red blood corpuscles were introduced in the reverse order. On testing heated plague serum mixed with plague bacilli and normal serum, Bordet and Gengou found that the subsequent addition of sensitized red blood corpuscles did not result in hæmolysis. The explanation they gave was that the sensitizing substance in plague serum fixes the complement in the presence of plague bacilli, so that it is prevented from acting on the sensitized red corpuscles. Other experiments of a like kind led the two Belgian workers to the belief that the phenomenon described had widespread applicability, and that it was possible to diagnose the presence of a ' sensitizer ' in the serum by the absence of hæmolysis of sensitized red blood corpuscles. Gengou (1902) showed further that ' sensitizers ' (amboceptors) are developed in the blood-serum of animals which have been injected with milk and that such sensitizers can fix complement. The same reaction was also found to occur in the sera of animals injected with egg albumin, fibrinogen and other substances. These facts were extended by C. Moreschi (1905) who found that complement fixation occurs in the presence of normal serum mixed with the serum of an animal injected with normal serum, and he demonstrated that extraordinarily small quantities of serum (0·00001 c.cm.) can be detected by this method of complement fixation. M. Neisser and Sachs (1905), in fact, recommended the method for the medico-legal diagnosis of blood. In 1906 Wassermann, A. Neisser and Bruck published their historic account, in which they described the discovery of antibodies to syphilitic antigen in the serum of syphilitic monkeys. In the same year Wassermann and F. Plaut (1906) by demonstrating syphilitic antibodies

in the cerebrospinal fluid of general paralytics proved this disease to be syphilitic. Wassermann, Neisser, Bruck and Schucht (1906) demonstrated similar antibodies in the blood-serum of syphilitic persons. Since that time the 'Wassermann test' has been practised to an enormous extent, and its principle has found applications in the diagnosis of glanders and other diseases.

The later ramifications of the immunity problem, the development of chemotherapy and the general advance of bacteriology are too recent to find a place in history.

From the small beginnings in 1675 when the great Dutch microscopist Antony van Leeuwenhoek first saw with his lenses minute living creatures in rain-water down to the end of the nineteenth century a new science developed and has now thrown out ramifications so numerous and intricate that the mind is bewildered, and relief is sought in an ever increasing specialization of knowledge. Among the subjects to be jettisoned by the modern worker is the story of the successes and failures of his predecessors —a story which is not always easy of access. I have, therefore, endeavoured to select from the original sources the dominating ideas of these men and to indicate the reaction of their contemporaries to these ideas, in the hope that the modern specialist may derive benefit from a study of the wider implications of the subject, or at least a warning of the benefits of humility.

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CHAPTER II. MORPHOLOGY.

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General Morphology.

The Schizomycetes or Bacteria are minute unicellular vegetable organisms, devoid of chlorophyll, and multiplying, in their normal vegetative state, by binary fission. They fall naturally into two divisions —the so-called 'higher bacteria', including the larger spirilla, the trichobacteria and thiobacteria, which exhibit well-marked filamentous and branching characters, and the simpler morphological forms or 'lower bacteria', with which we are here concerned and which we shall henceforth simply designate as bacteria. Certain bacterial groups, such as the corynebacteria and the mycobacteria may be considered as transitional forms connecting the higher with the lower bacteria.

Type Forms and Cell Grouping.

The bacteria comprise three fundamental type forms, spherical forms known as *cocci*, straight rods or *bacilli* and bent rods or *spirilla*. Though in the type form very distinct, they show intermediate forms that sometimes present difficulties in classification. Owing to the absence of chlorophyll nearly all bacteria are saprophytic or parasitic; most of them are colourless, but a few secrete colouring matter other than chlorophyll (e.g. *B. pyocyaneus*).

Cocci.

The cocci as a group exhibit characteristic differences in form. The pyogenic staphylococci and streptococci are isodiametric and present a globular structure, while many streptococcal forms are elliptical, and the *pneumococcus* is definitely lance-shaped. Division occurs either in one direction, whereby chain-formation takes place (streptococci) or in two directions at right angles, forming tabular connection (*Micrococcus tetragenus*) or into three-directional cubical packets (sarcinæ) or finally in three dimensions in space, but without regular arrangement (staphylococci). Among the staphylococci, however, short chains of organisms may be demonstrated, which show their relationship to the streptococci.

Bacilli.

In the typical bacillus the lateral surfaces are parallel to one another and in some forms (e.g. *B. anthracis*) the polar surfaces are at right angles to the sides. In many species, however, the ends are exteriorly rounded.

This polar convexity may be continued into the sides of the bacterium, so that they no longer remain parallel but assume an oval configuration (e.g. *B. pestis*), under certain conditions resembling streptococci. Spindle-shaped or fusiform bacilli are also found (e.g. the bacillus of Vincent's Angina). The diphtheria bacillus (*Corynebacterium diphtheriæ*) shows irregular deviation from the characteristic parallel-sided form, exhibiting club- and 'dumb-bell'-shaped growth forms. The plane of division is at right angles to the long axis of the bacillus and is never parallel to it. True branching occurs among certain groups of bacteria (corynebacteria, mycobacteria). The bacteria resulting from division may either lie free or exhibit a chain formation (streptobacilli). The position of the organism is sometimes characteristic, as in the case of the diphtheria bacillus, where the individual bacilli often lie in close parallel to one another or at various angles so as to resemble the letters V or L, or in combinations of these forms.

Spirilla.

According as the spirillum consists of a spiral of several turns or of only a section of a single turn, we speak of spirilla in the restricted sense, and of vibrios respectively. The latter are not only bent in one space-direction (as the familiar name 'comma bacillus' for the cholera vibrio might suggest), but are segments of a true spiral. Growth and division occur as in the bacteria, but on account of the active motility of these organisms, growth connections are seldom observed. *V. choleræ*, however, may exhibit 'S' connections between individuals.

According to Clark and Ruehl (1919), who made measurements of many species of organisms during growth, it is the rule for bacteria to lengthen during the first 6 or 9 hours, and then become rapidly shorter. *C. diphtheriæ* is, however, an exception in that it remains short for the first 6 hours of growth and only then begins to lengthen.

Size.

The size of the bacterial cell varies greatly according to the species. Among the largest met with may be mentioned *B. bütschlii* Schaudinn (1902), 4 to 5μ in width and some 50 to 80μ long, while among the smallest organisms, micrococcal forms exist with a diameter of only 0·2μ. The majority of bacteria and practically all the pathogenic forms occupy a mean position with regard to size: the staphylococci, for example, are about 0·5μ in diameter and the typhoid bacillus about 0·5μ in width by about 2 to 3μ in length. The spore-bearing organisms are as a rule somewhat larger; *B. anthracis*, for example, measures 0·75 to 1μ in diameter by 4 to 6μ in length, while *B. megatherium* is over 1μ wide and averages 8 to 10μ in length.

Pleomorphism.

Bacteria exhibit their characteristic morphology with regard to size and shape usually in young cultures and on media peculiarly favourable to their growth, but show departures from their standard morphology

under varying environmental influences. These aberrant types, which are fully described elsewhere (see Variation, p. 311), have been characterized as ' involution ' forms and are regarded by some authors as degenerating types. They are also considered by other observers to be definite stages in the life-cycle of the organism (see Life-Cycles of Bacteria, p. 170). As stated by Henrici (1928) it may well be that those factors which lead to the death of some of the cells in a culture may react upon others in such a way as to cause them to develop into one or another type of resting cell or reproductive body. Henrici prefers the term ' senescent forms ' to ' involution forms ' because the former clearly expresses the analogy between them and the cell changes which occur in old age in multicellular organisms, and because it does not so much imply that they are already actually dead.

The Cytology of Bacteria.

The cell contents consist of cytoplasm and ' nuclear ' material and are surrounded by a cell membrane, from the outer layer of which the capsule is derived. Under unfavourable environmental conditions, or perhaps as a regular stage in the life-history of the organism, many bacteria form by means of a concentration of ' nuclear ' material, spherical or ovoid bodies called spores, which exhibit higher resistance to injurious influences than the vegetative cells from which they arise. (For a description of the spore morphology in the resting cell and during the process of germination, see Cell Division, p. 165.)

In addition to spores other granular bodies are often present in the bacterial cytoplasm; these may perhaps be regarded as reserve food material or in certain cases as due to degenerative changes of a plasmolytic nature. Motility, when present, is always associated with the presence of flagella, locomotory organs attached to the periphery of the cell.

Cell Membrane and Cell Contents.

All bacteria possess cell walls which give definite contour to the cell, the bacterial body thus being divided into ' endoplasm ' and ' ectoplasm ' (Zettnow, 1897). The former, which comprises the cytoplasm of the cell, is believed to be more or less intimately mixed with the ' nuclear ' substance, or is arranged in a polar manner, and is stained blue with the Romanowsky stains. An ectoplasmic substance, on the other hand, remains unstained and only becomes visible after special preliminary treatment. The ectoplasm may be present in the form of a capsule which stains more feebly and takes on a pink tint with the above stains, but can be coloured more deeply by special methods. Flagella, when present, have been considered as ectoplasmic structures by some writers, but they probably are of quite a different composition to the capsule. They also require special methods in order to render them visible in stained preparations. This is in part due to their extremely small transverse diameter.

In some species of bacteria of large size the cell membrane may be distinguished when strongly magnified; among smaller bacteria it is scarcely visible and can only be observed when the cell contents have been contracted by plasmolysis or by a suitable reagent. Two layers have been recognized—an inner or cuticular layer, very thin and transparent, and an external or gelatinous layer (from which the capsule is derived) which results from a special differentiation of the peripheral zone of the inner layer.

The ectoplasm represents a relatively more concentrated substance than the endoplasm and in some species (e.g. *B. proteus*) comprises at least half the cell body (Zettnow, 1897). Unlike the cell walls of higher plants, it does not give reactions of cellulose, nor is chitin present as in the higher fungi. It consists of a protein substance and is apparently a modification of the general cell protoplasm. The appearances of the cuticular layer have been observed carefully by Schaudinn (1902) in the case of the cockroach bacillus—*B. bütschlii*—where it is clearly differentiated from the endoplasm and appears as a net-work with deeply staining refringent meshes. The ectoplasm has also been demonstrated by Heim (1889) in the case of *B. cyanogenes*, where, after plasmolysis with carbolic acid, the membrane is clearly seen in the form of a very fine contour. Gutstein (1925) has also succeeded in demonstrating the ectoplasm apart from the endoplasm of the cell by means of contrast staining and considers it to be the carrier of the Gram-fastness. He has also shown (1925[2]) that the ectoplasm of the Gram-positive bacteria contains a basic ground substance demonstrable by malachite green and a tannin mordant, and an acid body (lipoid) demonstrable by Victoria blue. Recently, Churchman (1927) has shown that *B. anthracis* consists of two distinct parts—a Gram-positive 'cortex' (ectoplasm) and a Gram-negative 'medulla' (endoplasm). The cortex, which may be removed by exposure to acriviolet or gentian-violet and even sometimes by hydrolysis in distilled water, is probably protein in character, since the Berkefeld filtrate of a suspension of *B. anthracis* exposed to gentian-violet gives a positive ninhydrin test. According to Burke and Barnes (1929) the cell protoplasm is not concerned with the Gram reaction. Varying degrees of cell-wall permeability are responsible for Gram-positive, Gram-negative and Gram-variable characteristics.

Nucleus or Nuclear Equivalent.

In spite of numerous researches extending over a period of some twenty-five years, the question of the presence of a nucleus among the bacteria is still a matter of controversy. In this connection it is well to bear in mind that we are almost entirely ignorant of the phylogeny of this group of organisms, and that observations which may be true for one species of bacterium may have no real reference to another. In other words, the group Bacteria, as at present constituted, may well comprise a very heterogeneous collection of primitive forms. The bacteria

are in no way a group of simple organisms, but display a marked degree of morphological differentiation which may be associated with a highly complex life-cycle.

Briefly to notice the extreme opinions on this subject of the bacterial nucleus: Migula (1896), Fischer (1903) and others contended that bacteria were very primitive organisms, devoid of nucleus and consisting simply of cytoplasm and vacuoles; granules, where present, representing products of nutrition. From purely theoretical considerations this view has since been considerably modified. Růžička (1908), on the other hand, maintained that the whole bacterial body should be regarded as a 'naked nucleus' analogous to the nucleus of higher organisms. Appearances suggestive of such a condition may be accounted for by an overstaining with the reagents employed, whereby a diffuse, reticular or even vesicular nucleus, if present, might easily fail to be differentiated from the cytoplasm of the cell. Moreover, as has been pointed out by Dobell (1911), there is good experimental evidence in other organisms that neither cytoplasm nor nucleus can exist independently one from the other for any length of time and that to call a bacterium a 'naked nucleus' is a misapplication of a term in common use. Most modern observers are agreed that bacteria do possess a 'nucleus', which may undergo marked modification in relation to the life-cycle of the concerned organism. A true vesicular nucleus, consisting of a colourless nucleoplasm surrounded by a membrane and containing karyosomes, has been described by Vejdovský (1900) in '*Bacterium gammari*', a species discovered by him in the body cavity and hæmolymph of the fresh-water crustacean *Gammarus zschokkei*. The same author (Vejdovský, 1904) discovered a similar structure in a filamentous bacterium found in the digestive tract of the annelid *Bryodrilus ehlersi*. His conclusions, although positive, are of doubtful value, in so far as the species described belong in all probability not to the bacteria but to the yeasts and higher fungi respectively. Mencl (1904) has endeavoured to apply Vejdovský's conclusions to other bacteria (e.g. *B. megatherium*), but his results are not convincing.

Guilliermond (1908) and other workers, while admitting that some bacteria may contain a more or less rudimentary nucleus, are of opinion that in the great majority of species nothing can be demonstrated other than a diffuse nucleus consisting of grains of chromatin scattered throughout the cytoplasm. This theory is in agreement with the investigations of Bütschli (1902), who described in certain of the higher bacteria (*Beggiatoa*, *Chromatium*) a central body of alveolar protoplasm containing numerous chromatic granules, the remainder of the cell consisting of a less easily stainable cytoplasmic layer surrounding the central body. Schaudinn's researches on *B. bütschlii* (Schaudinn, 1902)—a gigantic sporulating bacterium isolated from the intestine of the common cockroach—*Periplaneta orientalis*, also favours the diffuse nuclear theory. The bacterial cytoplasm of this organism consists of finely alveolar reticulate protoplasm containing many small chromatic granules. At the time of

sporulation these granules gather at the centre of the cell in an axial filament, two granular masses, from which the spores are ultimately derived, being formed eventually at either pole. Analogous conditions were also described by Schaudinn (1903) in a small marine organism (*Bacillus spironema*). These results have been confirmed by Guilliermond (1908) working with a large variety of endosporic organisms (*B. megatherium, B. mycoides, B. asterosporus, B. alvei*), who concluded that bacteria contain granular chromatin more or less mingled with cytoplasm, condensing at the time of sporulation to form the spore. The researches of Dobell (1911), who worked with micro-organisms obtained from the intestinal contents of frogs, toads and lizards, have given additional support to the presence of a diffuse bacterial nucleus, representing in his opinion the chromidial stage of the normal vegetative condition of the nucleus, existing throughout the greater part of its life-history. The staining methods employed were either fixation with osmic acid or formalin, followed by staining with one of the modifications of Romanowsky's method, or fixation with Schaudinn's sublimate alcohol (2 : 1) followed by staining with Heidenhain's iron-alum hæmatoxylin. He described a filamentous nucleus as a transient stage, connected with and immediately preceding spore-formation. In the spore itself the chromatin became densely aggregated and constituted the chief part of its living substance. In the process of germination of the spore the chromidial condition was again assumed. Thus spherical, filamentous and chromidial nuclei occurred in the same organism at different stages of its life-history. Dobell also observed, in certain *Micrococcus* forms, single centrally placed spherical bodies, which he regarded as nuclei. Similar appearances have been described by Nakanishi (1901) in *Staphylococcus aureus*. Douglas and Distaso (1912) demonstrated bodies resembling true nuclei in capsulated bacteria from the respiratory tract and Bessubetz (1925) on morphological grounds, and Schumacher (1926) on chemical grounds, also believe that certain bacteria possess true morphological nuclei.

Recently, Stoughton (1929) has demonstrated the existence of presumably nuclear structures in the plant pathogen—*Bacillus* (*Pseudomonas*) *malvacearum* by means of a special vital staining technique modified from the procedure of Nakanishi (1901). In these bacteria he has demonstrated the presence of a centrally placed body, staining with basic dyes, homogeneous and more or less spherical in immature organisms and becoming elongated and ' dumb-bell '-shaped in dividing cells (see Fig. 2, p. 174). He has observed a correlation between the division of this central body and that of the cell itself and expresses the opinion that the affinity for basic dyes, the position of the body in the cell and its shape and behaviour, all lend support to the hypothesis that these structures are of the nature of nuclei which undergo some kind of division. They are either true nuclei or possibly nuclei embedded in a mass of deeply staining reserve food substance, in accordance with the theory of Enderlein (1925). Stoughton has also observed these bodies, in living unstained organisms

growing on thin films of nutrient media, undergoing the process of division during the division of the cells themselves. Each half of the central structure was seen to pass into the respective daughter-cells. On account of the limitation of optical resolution the details of the central body could not be ascertained, but the evidence for the presence and division of the central structure coincident with the division of the cell, was deemed conclusive.

Stoughton has also demonstrated other granules, distinct from the central (' nuclear ') structures which stain very early and very intensely in wet film preparations with basic dyes. They are formed, adpressed to or actually in the cell wall and are finally liberated either by simple extrusion through the wall or else by growth on an elongated stalk, which may reach a length of 1 to 2μ ; the granule appears to be invested with a membrane or sac continuous with the stalk. He regards these granules as ' gonidia '—organs of asexual reproduction in the sense employed by Löhnis (1921)—despite the fact that they have not yet been observed to grow out into normal rods.

Reserve Material.

Metachromatic Granules.

In addition to nuclear material, various granular bodies may be contained in the bacterial cell, which do not exhibit the characters of chromatin. They assume a reddish-violet colour when stained with methylene blue and certain other dyes. They have been confused with chromatin on account of their affinity after fixation for so-called ' nuclear ' stains, and have been repeatedly observed and described under such names as ' metachromatic bodies ' (Babes, 1895), ' sporogenic granules ' (Ernst, 1888), ' volutin granules ' (Meyer, 1904), ' polar granules ' and ' Neisser's bodies '. The metachromatic granules (also called Babes-Ernst bodies after their first discoverers) occur in single or multiple thickenings of the protoplasm, varying in number from a single one, situated at one end, to four or even five distributed along the whole length of the bacillus. They are remarkable in the unstained condition through their strong refractility ; in their stained condition through their strong affinity for basic aniline dyes, which they take up readily and part with with difficulty. By means of double staining (methylene blue-vesuvin or carbol-fuchsine-methylene blue), Ernst and Neisser (1888) were able to demonstrate these granules in distinct colours inside the cell. They may also be demonstrated by vital-staining, the possibility that they are artefacts being thus excluded. Chemically considered, the metachromatic granules are properly to be regarded as composed of nucleic acid combined with an organic base (Guilliermond, 1906).

Various theories have been advanced as to the nature of these bodies. For example, it was held by Marx and Woithe (1900) that they were an index of the virulence of the containing organism, but in view of the researches of Ascoli (1901) and others and the clinical observations of

Schumberg (1902), this view cannot be maintained. Nor can they be regarded as ' sporogenic ' granules, as at first believed by Ernst himself, for, as has been pointed out by subsequent observers, they are destroyed by boiling methylene blue (by which immature spores are stained) and also by boiling water.

There does not, however, appear to be any real question as to their biological significance. They are not living morphological derivatives of either nucleus or cytoplasm, but merely stored up reserve material and are transient constituents of the cell. They tend to disappear during active growth and to reappear after growth has ceased, and are found in varying numbers in different species of bacteria, conditioned by the age of the cells and the nature of the surrounding media (*C. diphtheriæ*, *B. volutans*, *B. asterosporus*, *B. alvei*). They appear in cultures 4 to 8 hours old in the form of small grains, which gradually increase in size, reaching, in the case of *C. diphtheriæ*, their largest average size in from 12 to 15 hours. After this period they diminish in size and decrease in number, and at the end of two days most of the bacilli have lost their granules (Albert, 1921).

According to Babes (1910), metachromatic granules are also met with in the acid-fast group of bacteria and are identical with the granular forms of the tubercle bacillus described by Much (1907), and believed by Fontes (1910) to represent a filter-passing stage.

In addition to the true metachromatic granules, other reserve products, such as globules of fat, glycogen or starch granules, have occasionally been observed. The starch or iodophile granules, which stain blue with iodine, may be formed even when the bacterium is growing on a starch-free medium. Among the sulphur bacteria numerous fine grains of sulphur may be scattered throughout the cytoplasm.

Capsules.

Mucilaginous envelopes, described as capsules, are probably present, if only in small grade, among all bacteria. They are particularly well marked among certain species, notably the *Pneumococcus* of Fraenkel, Pfeiffer's Capsule Bacillus (*B. lactis aerogenes*) and *B. welchii*—the *B. aerogenes capsulatus* of some writers. In preparations stained in the ordinary manner with basic aniline dyes the capsule appears in the form of a bright halo surrounding the bacterial body, but its definite structure is only revealed by means of special capsule stains or by dark ground illumination. In connection with staining methods it must be observed that a too energetic procedure may lead to the production of artefacts, and to the demonstration of ' false capsules ' around organisms not usually considered to be capsule-bearing (Boni, 1900).

Capsules are formed from the external or gelatinous layer of the bacterial ectoplasm and with bacteria which exhibit characteristic growth connection, such as the *Pneumococcus*, the associated organisms are surrounded completely by a common capsule. According to Fürst (1910),

Heim (1904) and Toenniessen (1912) the capsule may be differentiated into two layers, the inner representing the vital layer belonging to the ectoplasm, whilst the outer is merely a secretion product.

Bacteria such as *B. anthracis*, which show characteristic capsules in animal secretions, may completely lose this peculiarity in artificial culture. With the plague bacillus, on the other hand, growth in appropriate media, such as horse-serum, actually favours their production (Rowland, 1911). In others (e.g. *B. welchii*) where the capsule is always present, it cannot be equally well demonstrated in all culture media, but may be made evident by transplanting the organism into serum or weak sodium hydrate solution (Robertson, 1916).

In the above-mentioned species the capsule is to be regarded as an integral part of the organism under normal circumstances, but in addition, capsules are often formed and deposited on bacteria under special conditions of growth. *B. subtilis* and *B. mesentericus*, for example, may become encapsulated when growing in the intestinal contents or in bread dough or similar material (Vogel, 1897) : such structures are rapidly lost on artificial cultivation. Similarly *Streptococcus* (*Leuconostoc*) *mesenteroides*, which is found in the syrup of sugar refineries, forms abundant capsules in this environment. The organism changes the character of the syrup and its presence and growth is often the cause of serious economic loss. The slime formed by these zooglœal masses of organisms protects them against syrup which has been heated to temperatures as high as 80 to 85° C. (Orla-Jensen, 1919). The capsule of the anthrax bacillus has obtained special diagnostic significance in that the various organisms described as ' anthracoid ' or ' pseudo-anthrax ' bacilli do not usually possess this structure (Vol. V, Chapter XI).

The fact that capsules are developed more strongly among certain, especially among infective, organisms, was first recognized by Babes (1895) as showing that their formation is a defensive activity of the bacterial body when threatened by exterior forces inimical to microbic life. Capsule formation is generally considered not as a degenerative process, as formerly assumed by some authors (Pane, 1898), but as an active reaction of the bacterial cell, accompanied by an increase of virulence and capacity for resistance against bactericidal serum (Bail, 1915) as well as against phagocytosis (Bordet, 1910), and is aptly described by Sauerbeck (1909) as bacterial immunity through structural adaptation. As observed by Rowland (1911) the plague bacillus under certain conditions develops a true capsule, demonstrable by means of the usual capsule stains ; but he has also shown the presence of a slimy envelope, without definite outer limit and of viscid consistency. This condition is probably of a degenerative nature ; it contributes to the viscosity of plague cultures on solid media (Gotschlich, 1927), and is comparable to the so-called ' lepra-slime ' described by Unna (1888) in human tissues.

In recent years more regard has been paid to the chemical composition of the capsular substance of the true encapsulated bacteria. Toenniessen

(1921), for example, has shown in the case of Friedländers's bacillus that the secreting capsule is free from protein and contains galactan and a polysaccharide of galactose. These results were later extended by Kramár (1921), who demonstrated a glycoprotein in the capsule of *B. anthracis* and a dextran in that of *B. radicicola*. Avery, Heidelberger and Goebel (1925) have isolated a capsular substance of the Type II *Pneumococcus*, and find it to be a polysaccharide. This substance was found to be non-antigenic, while the protein substance derived from the bacterial body was antigenic but had no type specificity. In order to obtain type-specific reactions in animals it was found necessary to employ both the protein constituent of the cell body and the capsular substance before separation of the cell mass into its constituents.

Flagella.

Flagella, the motile organs of bacteria, first demonstrated by Cohn (1875) and Koch (1877) in the larger spirilla, are narrow thread-like structures. They cannot be seen without staining or special optical methods, such as dark ground illumination, by which latter they may be demonstrated in the living condition and in full movement. They appear only to be accessible to staining reactions after the preliminary application of a mordant, as first suggested by Loeffler (1890). They may also be demonstrated by the silver impregnation method of van Ermengem (1894), which possesses great similarity to certain photographic procedures. This phenomenon is not simply one of silver precipitation on the bacteria and flagella, but is due to a true chemical combination, indicating a different microchemical structure between flagella and bacterial bodies.

The stained flagella appear as slender, long (up to 32μ), undulating, flexuous threads that end bluntly—often indeed with thickened ends. The behaviour of the flagellum has been investigated by Reichert (1909) and Metzner (1920), who amongst various species of flagellated bacteria examined find that, when viewed from behind, they are right-turning rotary spirals, rotating in the opposite direction to the bacterial body. The flagella are always directed backwards, equally whether they originate from the front or back pole. According to Fildes (unpublished) the flagella of *B. tetani* and *B. proteus* are parallel and directed backwards at an angle of about 45°. The forward movement is due to spiral undulations of the flagella. Reversal of direction is obtained by swinging all the flagella through an angle of some 90°, or turning movements by directing forward only a few of the anterior flagella on one side to produce a lateral bias to the leading end. With many organisms (especially *B. chauvœi* and *B. tetani*) flagella, which have been torn from the parent cell, become associated in massive plait-like structures ('Geisselzöpfen'), considerably exceeding the relative bacteria in size and sometimes, indeed, visible in the unstained condition. Noguchi (1926) has shown by dark-ground illumination methods, that spirochætes

belonging to the *Spironema* and *Treponema* groups produce under certain cultural conditions exaggerated flagellar appendages, similar to, but much finer than, the axial spiral filaments of these organisms. The axial filaments are covered with a cytoplasmic layer which can be removed by the action of bile. Motility resides in the portion of the filament next the attachment of the flagellum at either end. The great resemblance which exists between the flagella of motile bacteria and the flagellar and axial spiral apparatus of these spirochætes seems to indicate that the axial filaments are probably a modified apparatus of similar origin, especially adapted to the locomotion of spirochætes, and, therefore, supports the hypothesis of a close phylogenic relationship between bacteria and spirochætes.

The number and arrangement of the flagella is different among individual kinds of bacteria, and, broadly speaking, is constant with each individual species. In consequence of this, the flagellation of bacteria may serve as a differential diagnostic characteristic. For example, the following scheme of classification dependent on flagellar characters has been erected by Messea (1896) :

Atricha. No flagella. Non-motile bacteria (e.g. *B. anthracis*).

Monotricha. A single flagellum at one pole (e.g. *V. choleræ*).

Amphitricha. A flagellum present at each pole (e.g. numerous *Vibrio* species).

Lophotricha. A tuft of flagella at either or both poles (e.g. Larger Spirilla).

Peritricha. Flagella in varying numbers originating from the sides of or distributed around the bacterial body (e.g. *B. typhosus, B. proteus*). With the latter organism the flagella are so numerous that they form a thick, felted texture.

There is considerable diversity of opinion as to the structural origin of the bacterial flagella. Some considerations point toward their derivation from the bacterial ectoplasm ; their chemical differentiation from the endoplasm in van Ermengem's silver impregnation method would appear to support the view that they represent especially differentiated parts of the ectoplasm, and their direct origin from the bacterial envelope or capsule, as the case may be, has been observed by many workers (Babes, 1890, and others). Their antigenic structure also shows them to be sharply differentiated from the cell protoplasm. Fischer (1894), on the other hand, considered the flagella to be prolongations of the cytoplasm, issuing through apertures in the cell wall, while Fuhrmann (1909) and Yamamoto (1910) are of opinion that the proximal end of the flagellum stands in direct relation to a chromatin granule in the cell body, corresponding to the blepharoplast of the protozoa.

R. St. J.-B.

Morphology of Bacteria in the Living State.

By J. E. Barnard.

The literature on the subject of bacterial morphology is so extensive and the interpretation of such structure as staining methods demonstrate so variable, that no effort will be made here to summarize the results so far obtained. It is, however, the general practice to rely on such staining methods for morphological investigations applied to presumably dead cells or to cells that have been ' fixed ' in some way, and that are, therefore, no longer carrying on any part of their life process. The purpose of this short article is to draw attention to the structure that can be demonstrated in some bacteria while they still live, although it must be admitted that it is not always easy to secure all the conditions for the exercise of full reproductive powers, and at the same time to be able to observe such processes under the microscope. The method by which the photo-micrographs accompanying this article have been produced will, therefore, be described briefly, together with such cultural details as are essential to the photographic method.

For the microscopical observation of living organisms the ' dark-ground ' illuminator is the most suitable, and, providing that this appliance is properly used, particularly that perfect centration to the optic axis of the microscope is assured, it does provide a satisfactory means of observing even small bacteria. Its limitations, however, soon become evident, partly because observations must be made in a watery medium of low refractive index and also because, as the result of this factor, objectives of relatively low aperture must be used for observational purposes. Recently considerable advances have been made in the use of ultra-violet light for dark-ground illumination purposes, and it is the results so obtained that are reproduced here. The method cannot be described in detail, but it consists essentially in illuminating living organisms by an annular beam of ultra-violet light, in principle the same as in visual work, and photographing the invisible image formed, by a suitable quartz objective.

The type of illuminator now used differs somewhat from that most suitable for visual light ; it is made of quartz and magnalium to secure sufficient transmitting and reflecting power in that portion of the ultra-violet spectrum that is being used. The results so obtained do, however, suggest that the morphological details and internal structure can be shown with greater certainty than by any other process. The delicacy and fineness of some details can be observed only in the original photographs or negatives, no reproduction process can do full justice to them.

The cultural methods employed have in each case been those generally regarded as most suitable for the particular organisms selected. In some cases photographs have been taken of colonies *in situ* on quartz slides. These are in general the most suitable for the purpose, providing that the layer of nutritive material is thin and free from visible impurities.

Organisms grown on serum-agar are often difficult to deal with owing to the opacity of such media to ultra-violet light, but by keeping the layer of medium very thin this difficulty, particularly in the case of bovine pleuro-pneumonia, has been overcome.

The photo-micrographs are arranged approximately in order of size, the larger organisms being on Plate I, and the smaller, probably showing contents of the order of size of some viruses, on Plate II. There would only appear to be one morphological difference between large and small micro-organisms, regarding them solely in terms of size, and that is the nearer approach to sphericity possessed by the smaller ones. This is a fairly common property of small plastic bodies, whether living or non-living, and may be due to the high surface tension of such small objects. There is also definite evidence that the smallest observable organisms have greater density and consequent greater visibility than larger ones, a point of considerable interest that can hardly be dealt with here exhaustively. It does, however, tend to increase the difficulty of observation as it results in apparently increased thickness of cell wall with consequent lower visibility of cell contents. In a young growing cell, one in which subdivision is only just completed, the colloidal constituents of the protoplasmic contents of the cell appear to be in high state of division, granules of observable size are few and not easily seen. These increase in number with age, and are at a late stage difficult to distinguish and cannot be differentiated from nuclear bodies. The first stage in reproduction appears to be a separation of the clear cell contents into two polar masses, shown best, perhaps, in Plate II, Fig. 5. A clear space is left between, no separating membrane forms until a later stage when the cell wall becomes indented at the position where subdivision is obviously commencing. Such structure as it is possible to demonstrate is shown in Plate I, Fig. 1, *B. megatherium*, the highly refractile spores standing out clearly from the granular cell contents. That bacteria possess a nucleus which undergoes mitotic division is clearly suggested in Figs. 2 and 3, Plate I. A comparison of Figs. 8 and 9, Plate II, is even more suggestive. Fig. 8 is a dark-ground picture taken by the method herein referred to, whereas Fig. 9 is the fluorescent image, the object still being illuminated by ultra-violet light, taken by means of an ordinary oil immersion apochromatic objective. The result is that the image obtained shows only those granules that fluoresce, and the suggestion is that nuclear bodies respond differently to the other granular contents of the cell. The argument cannot be followed further at present as the method is still experimental and in course of development.

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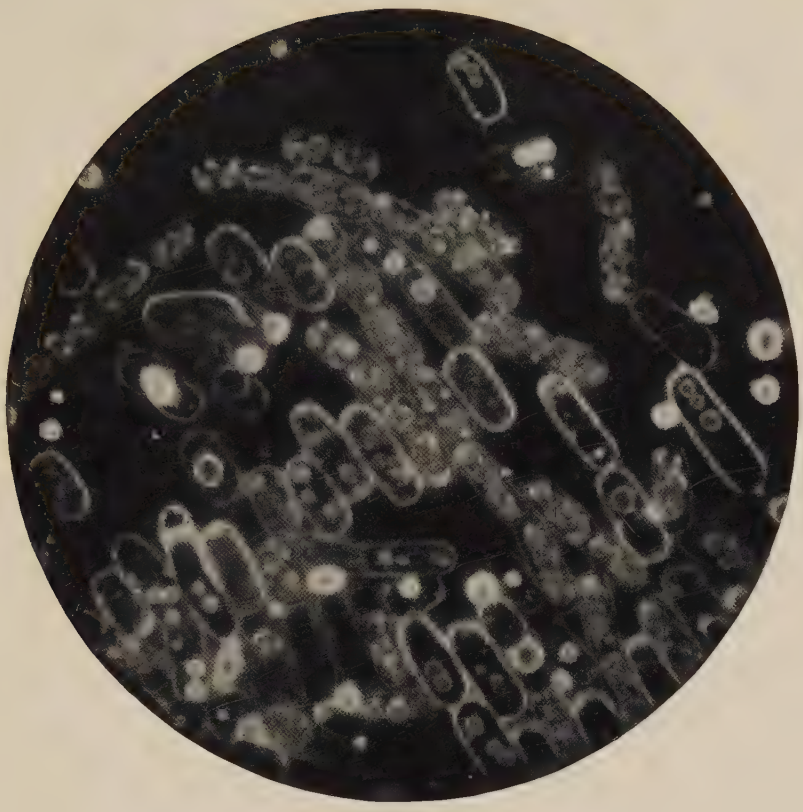

FIG. 1.—*B. megatherium* × 4,000. 24 hrs. agar culture at 37° C.

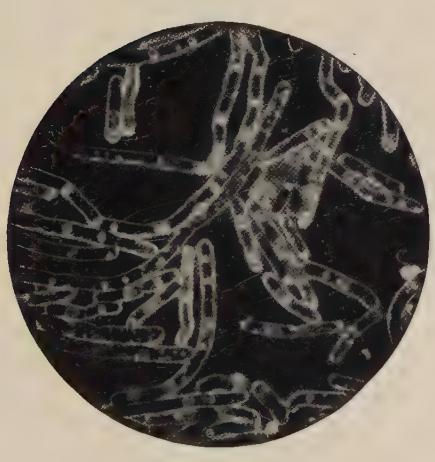

FIG. 2.—*B. mycoïdes* × 2,000.
24 hrs. agar culture at 37° C.

FIG. 3.—*B. megatherium* × 2,000.
24 hrs. agar culture at 37° C.

[*To face p.* 116.

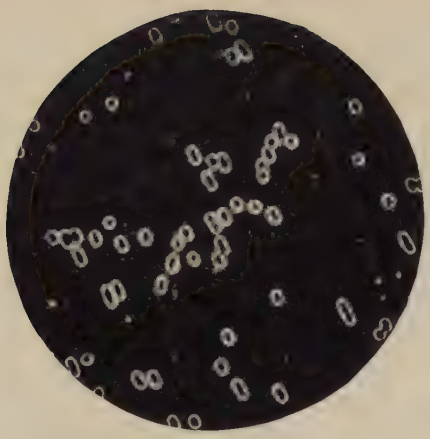

FIG. 4.—*B. bronchisepticus* × 2,000.
24 hrs. agar culture at 37° C.

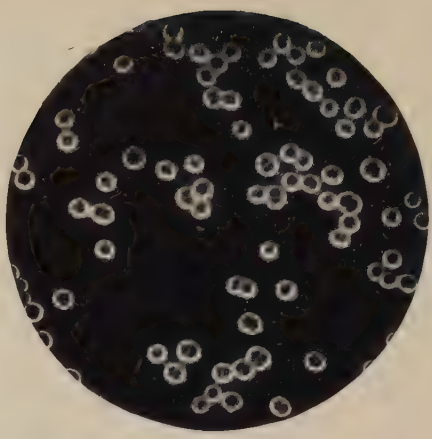

FIG. 5.—*M. pyogenes aureus* × 2,000.
24 hrs. agar culture at 37° C.

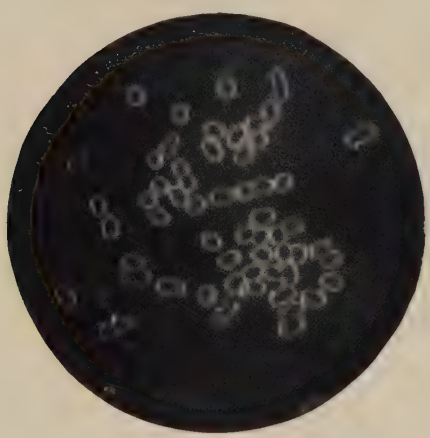

FIG. 6.—*B. coli* × 2,000.
24 hrs. agar culture at 37° C.

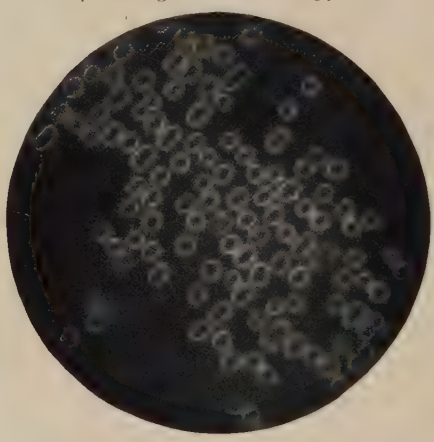

FIG. 7.—*B. prodigiosus* × 2,000.
24 hrs. agar culture at 37° C.

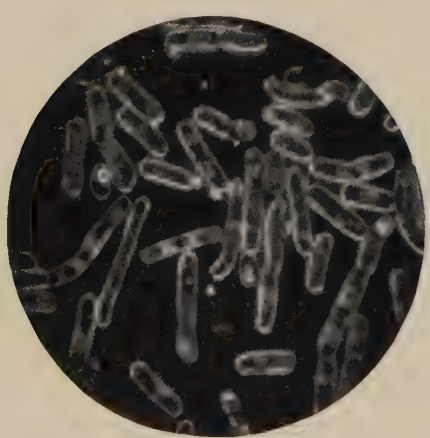

FIG. 8.—*B. megatherium* × 2,000.
24 hrs. agar culture at 37° C.

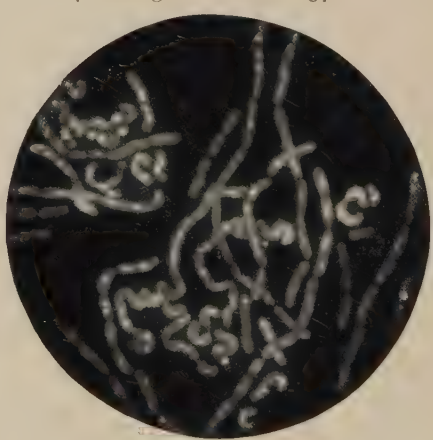

FIG. 9.—*B. megatherium* × 1,200.
Fluorescent image.

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CHAPTER III. THE PHYSICS OF THE BACTERIAL CELL.

By E. K. Rideal (University of Cambridge); with a Section by Hamilton McCombie (University of Cambridge).

Osmosis.

If a solution be brought into contact with the solvent, diffusion of solute into the pure solvent and of solvent into the solution occurs; on separating solvent from solution by a membrane permeable to the solvent but impermeable to the solute, the diffusion of the solute into the solvent can no longer occur, but the diffusion of the solvent into the solution still continues; this phenomenon is termed osmosis. We may apply a differential pressure between solution and solvent, such that the rate of flow of the solvent from the solution through the membrane into the solvent under the influence of the pressure gradient is exactly equal to the osmotic flow of the solvent in the reverse direction into the solution. This pressure is termed the osmotic pressure of the solution.

The laws of osmotic pressure for dilute solutions developed by van't Hoff may be summarized by the following expression:

$$\pi = \frac{N}{V_o} RT$$

where π is the osmotic pressure exerted by a solution containing N particles of solute in V_o volumes of solvent at a temperature T, R being the gas constant. It is frequently stated that osmotic pressure is caused by molecular bombardment of the solute particles on the one side of the semi-permeable membrane, but from what has been stated as to the origin of the osmotic flow this is not the case. This point is clearly exemplified by consideration of a molar solution of cane-sugar placed in a vessel with one wall consisting of a semi-permeable membrane; the pressure due to the sugar molecules on the wall is but a fraction of one atmosphere, but the osmotic pressure necessary to apply to the sugar solution to maintain equilibrium of the water on each side of the semi-permeable membrane is some 22 atmospheres. The simple 'gas law' expression stated above does not hold true for strong solutions. Numerous investigators, notably Porter and Sackur, have attempted to arrive at more exact expressions by modification of the van't Hoff expression on the lines of equations of state such as that of van der Waals for gases under high pressure. The legitimacy of such an operation may be questioned, but an empirical equation of the following type expresses the results for non-electrolytes to a high degree of accuracy:

$$\pi = \frac{N}{V} RT \left\{ 1 + \alpha \frac{N}{V} + \beta \frac{(N)^2}{V} + - - \right\}$$

where V is the volume of the solution, and α and β are constants.

It is frequently inconvenient to measure the osmotic pressures of solutions directly, but it is possible to calculate their magnitude from a knowledge of the fact that the vapour pressure of any component of a liquid phase is increased when the external pressure on the liquid phase is increased. We must note, however, that on increasing the pressure on the solution, we increase the pressure not only on the solvent, but on the solute as well. It is convenient in many cases to neglect this fact, i.e. to treat the vapour pressure of the solute as independent of the pressure on the solution, and we can then obtain a relationship (necessarily inexact) between van't Hoff's law and Henry's law which states that the vapour pressure of any chemical substance present in a solution is proportional to its molar fraction.

We have noted that in applying a suitable pressure to the solution on one side of a semi-permeable membrane the solvent in the solution is brought into pressure equilibrium with the solvent outside. The solutions are likewise in thermal equilibrium and the vapour pressures of the solvent above solution and solvent will likewise be identical. We may, in fact, define the osmotic pressure of a solution as that pressure which it is necessary to apply to the solution in order to raise the partial vapour pressure of the solvent therein to that exerted by the pure solvent under one atmosphere pressure at the same temperature. It must be observed that this definition is not phenomenological but derived. It is, however, a convenient one, as it establishes a thermodynamic connection between the osmotic pressure, and the other so-called colligative properties of a solution, such as the lowering of the vapour pressure and freezing-point and elevation of the boiling-point. The thermodynamic relationship between the vapour pressure of the solvent above a solution and the osmotic pressure of the solution is expressed in the form

$$\left(\frac{dp}{d\pi}\right)_T = -\frac{V_o}{v}$$

where v is the molar volume of vapour at a pressure p and temperature T, and V_o the molar volume of the solvent at T and at an osmotic pressure π. This may be written in the form $v\ \delta p = -V_o \delta\pi$, and if we assume that the vapour obeys the perfect gas laws we obtain $RT\ \delta \log p = -V_o\ \delta\pi$ which for an uncompressible liquid (V_o = constant) can be integrated in the form

$$\pi V_o = RT \log \frac{p}{p_o}$$

$$\text{or } \pi = \frac{RT}{V_o} \log \frac{p}{p_o}$$

By Henry's law we obtain $\delta p = K\ \delta N$, where N is the molar fraction of the solvent. If Henry's law holds true over the whole range of concentrations examined we may consider K as a constant and obtain $p = K\ N$. Eliminating K we obtain $\frac{\delta p}{p} = \frac{\delta N}{N}$.

Since $RT\ \delta \log p = -V_o\ \delta\pi$ we obtain $RT\ \delta \log N = V_o\ \delta\pi$, and integrating this expression with the same limitations as above we find

$$\pi = \frac{RT}{V_o} \log_e N *$$

We have thus derived the formal relationships between the osmotic pressure of a solution and the molar concentration of the solvent as well as the partial pressure of the solvent vapour.

Membranes. We have noted that an osmotic pressure can only be developed when a solution is separated from another of different isotonicity by a semi-permeable membrane. From the theoretical point of view, the nature of the membrane is immaterial, provided that it is impervious to the osmotically-active constituents of the solution, but permits passage of the solvent. Again the mechanism by which the solvent passes the membrane is not material, provided that over all the ranges of concentration examined the process of transfer from one side to the other is perfectly reversible.

If a solution of a non-volatile solute be separated from the solvent by a gas space, distillation can occur through the space and the phenomenon of osmosis takes place across the gas membrane. If the gas be replaced by a liquid in which the solute is not soluble but the solvent is soluble, the same phenomenon is noted. Thus the material which constitutes the active portions of a semi-permeable membrane (for in general membrane support material whether organic or inorganic constitutes a considerable fraction of both synthetic and natural membranes) need only possess the property of being able to dissolve solvent for osmosis to occur. Most membranes are by no means ideal ; whilst certain solutes exert and maintain the theoretical osmotic pressure for long periods others slowly pass through the membrane, whilst others again pass through as rapidly as the solvent. It is natural to associate these differences in plasmolytic properties of various solutes with differences in their solubilities in the membrane material just as osmotic phenomena across a gas membrane are modified with solutes possessing finite vapour-pressures.

It so happens, however, that membranes are not so simple structures, and this idea has to be supported by somewhat arbitrary hypotheses. Thus, in the most suitable inorganic membrane, viz. copper ferrocyanide, we find that it consists of a network of hydrated gel-like salt supported by the earthenware or other porous support material. Such a membrane is permeable to water but not to sugar. On treatment with substances such as alcohol or strong salt solution the membrane is now permeable both

* In place of the molar fraction of solvent N we may write $1 - N_A$ where N_A is the molar fraction of the solute. Expanding $\log_e (1 - N_A)$ by Maclaurin's theorem we obtain

$$\pi = \frac{RT}{V_o} \left(N_A + \tfrac{1}{2} N_A^2 + \tfrac{1}{3} N_A^3\right) = \frac{RT}{V_o} N_A$$

for small molar fractions.

to solvent and to the sugar. Investigations on colloidal copper ferrocyanide reveal the fact that this change in permeability is associated with a conversion of the colloidal gel-like particles into a coagulated or crystalline precipitate. We may with some degree of certainty conclude that the property of semi-permeability in this membrane is associated with the gel form. The gels consist in general of fibrils of solvated gel-forming material intermeshing with one another in irregular distribution to form a felt-like mass enclosing in the interstices a saturated solution of the sparingly soluble gel-forming material. Through such a gel the solvent can diffuse without difficulty, but the penetration of a solute will be dependent on a number of factors. In the case of a membrane separating two liquids, e.g. water and alcohol, or water and pyridine, diffusion across it of either liquid will clearly depend upon its nature, thus rubber forms a gel readily with alcohol and pyridine, and the membrane is according permeable to these liquids but not to water; with parchment, bladder and copper ferrocyanide membranes the reverse is the case. The direction of flow of liquid will thus be dependent on the nature of the membrane and of the liquid.

Since the solute has to pass through the interstices or pores in the felt, such membranes may be regarded in part as sieves, in that the pore diameter greatly affects the rate of passage of the solute. This sieve-like* character is predominant in certain membranes such as dried collodion, in which the collodion aggregates are by no means solvated to such an extent as the fibrils of the copper ferrocyanide membrane. Through such purely sieve-like membranes solutes diffuse at rates varying almost inversely with their molecular volume, which are in the same order as the diffusion rates in the free solution. When the solute is an electrolyte, on the other hand, several factors have to be taken into consideration. In the first case membranes are in general not electrically neutral, they are usually charged. This charge may be acquired either by surface ionization or by selective adsorption of ions from the solution. If the surface of the membrane possesses a charge brought about by either of these causes an equal and opposite ionic charge will be maintained in the neighbourhood by electrostatic attraction, thus forming the so-called electrical double layer of Helmholtz. The electrical double layer present on the fibrils or cell-walls of membranes exerts a profound effect, both on the swelling of the membrane and on the rate of passage of electrolytes through the membranes. Michaelis found that with fine collodion membranes the diffusion of potassium chloride into water barely took place, but if the water was replaced by a solution of sodium nitrate interchange of the cations across the membrane occurred. If solutions of $\frac{N}{10}$ and $\frac{N}{100}$ KCl

* Bartell (1912) showed that with membranes of aggregated silica and carbon osmotic effects were first obvious when the computed pore size was 900$\mu\mu$. Tinker (1916) showed that the average pore size in a $Cu_2Fe(CN)_6$ gel membrane was 15 to 20$\mu\mu$, an indication that adsorbed water must play a part in restricting pore size.

are separated by a collodion membrane a potential difference is obtained, due to an increase in the relative mobilities of cations and anions when diffusing through the membrane. Collodion acquires a negative surface charge as shown by cataphoresis experiments, i.e. the anions are anchored on the pore walls and diffusion is mainly confined to the cations. The enhanced relative mobility difference between anion and cation is thus probably due both to an increase in the cation mobility, u, and to a decrease in the anion mobility, v, in the pores of the membrane. The net effect is shown in Table I.

TABLE I.

	+ Li	+ Na	+ K	+ Rb	+ H
Relative mobility within collodion membrane.	0·048	0·14	1	2·8	42·5
Relative mobility in aqueous solution ..	0·52	0·65	1	1·04	4·9

With protein membranes a similar effect can be noted. If a gelatin membrane separating the two salt solutions be brought to its iso-electric point by adjusting the solutions to pH 4·7 the double layer disappears and the potential difference between the solutions may be expected to be identical with that for a pure sieve membrane. If, however, the double layer be formed either by the addition of acid or alkali, the potential differences due to alteration in the relative velocities of ionic migration should make their appearance (Fujita, 1925; Hober, 1924; Matsuo, 1923; Mond, 1924).

With such membranes separating two solutions of electrolytes of unequal concentration there clearly exist several sources of potential differences, the Nernst potential difference between the two solutions separated by the membrane, and the Helmholtz double layer or Freundlich potential difference. Since this latter is caused by surface ionization or selective ionic adsorption its value will be different on each side of the membrane. According to Girard (1908, 1928), Bartell (1914) and Loeb (1919), when these conditions are established negative osmosis or apparent migration of the liquid from the dilute to the strong solution may take place.

Osmotic Phenomena in Bacteria.

In general, bacterial cells may be regarded as a chromatin network in an emulsoid protoplasm, the endoplasm, the whole being contained in a semi-permeable envelope, the ectoplasm. Whilst some organisms, e.g. *C. diphtheriæ* contain celluloses or hemi-celluloses, and many contain chitin and pectin, the ectoplasm is probably not mainly cellulosic as in plants, but is complex, differing chiefly from the endoplasm in being poorer in water. Owing to their minute size, differential analysis of cell wall and contents is not a simple matter. Murray, effecting a separation of the

cell walls of *C. dysenteriæ* from the contents by osmotic methods, obtained the following analytical data :

	Dried Bacilli.	*Cell Walls* (55·4 *per cent. by weight of cells*).
Protein	79·56	88·02
Fats	3·45	1·44
Water	3·60	0·91
Carbohydrates ..	5·28	7·94
Ash	8·11	1·69

A partial analysis of the cell walls of myxomycetes reveals some 4 per cent. of ether-soluble material, 5 per cent. of complex carbohydrates, and 3·63 per cent. N as protein. This difficulty of analysis is accentuated by the fact that although in some organisms, e.g. *B. cyanogenes*, the membrane is sharply distinguished from the endoplasm ; in others, e.g. spirillæ it is scarcely differentiated.

That such cell walls do exist and behave as semi-permeable membranes is observed from the fact that there is normally an osmotic or turgor pressure of the cell contents when the cells are immersed in water. The turgor pressure is usually low, lying between one and ten atmospheres. Insertion of many bacteria, e.g. *V. choleræ*, *B. typhosus*, *B. pyocyaneus*, in hypertonic solutions effects plasmolysis, and the reverse phenomenon of swelling or plasmotysis (in some cases even to bursting) can also be effected. We may note that the rate of ingress or egress both of salts and of water varies widely with the nature of the organism and is in general rather slow, thus limiting, as Murray has shown, the utility of the plasmolytic method for the recovery of endotoxins. It is found as anticipated that blood-cells and bacilli can be burst by these methods more readily than spherical cocci.

It may be observed that the conditions of equilibrium in a small cell are governed not only by the turgor pressure of the cell contents but also by the interfacial surface energy of the elastic membrane. For small cocci the excess pressure due to this cause is given by the expression

$$P = \frac{2\sigma}{r}$$

where σ is the interfacial energy and r the cell radius. For cocci of radii $0{\cdot}1\mu$ and $1{\cdot}0\mu$ respectively with the interfacial energy of 25 ergs per square cm. the excess pressures will be some 5·0 and 0·5 atmospheres respectively.

We note the evidence for a membrane in micro-organisms, that it behaves in part as a semi-permeable membrane, and that it is undoubtedly complex in composition. The investigations of Hamburger, Overton and Lowe (1884), and Overton and Meyer (1901), Herzog and Betzel (1910, 1911), Gössl (1913), have shown that narcotics are usually lipoid-soluble, and in spite of the obvious criticism that lipoid-soluble substances may pass into cells by destruction of the envelope it is reasonable to assume

that lipoids form part of the cell membrane of most bacteria. Fats are likewise present, as detected by fat-soluble dyes, e.g. Sudan III, or by oxidation with osmic acid ; tubercle bacilli containing as much as 10 per cent. of fat by weight in the dried material. In blood-cells the investigations of Gortner and Grendel have shown that the lipoids, chiefly cholesterol and lecithin, are present in quantities sufficient to form a bimolecular layer. In soap bubbles likewise the elementary leaflet consists of two planes of unimolecular layers of soap with their carboxyl heads facing one another, enclosing, as it were in a sandwich, a certain amount of water. These apparently uniform molecular distributions may likewise obtain in the membranes of bacteria.

We are indebted mainly to Overton for the correlation between the ease of penetration into cells and the lipoid-solubility and non-polar character of the diffusing substance. Thus the hydrocarbons penetrate with great ease ; but insertion of polar groups, e.g. — COOH — NH_2 — OH, into the hydrocarbon molecule slows down the rate of penetration. The effect of polar groups is very marked, as can be exemplified by a comparison of the rates of penetration of a series such as ethyl alcohol, glycol, glycerol, erythritol and mannitol. Ethyl alcohol penetrates with ease, glycerol very slowly ; only slight penetration can be observed in erythritol, and no observable rate of penetration can be noted in the case of mannitol. In spite of this it is clear that the sugars must enter the cells, although they do so extremely slowly (Brooks, 1916). We shall observe, however, that the factor of membrane permeability is affected by an alteration of the surface tension, which in turn is affected on adsorption, thus sugars and amino-acids (Höber and Memmesheimer, 1923) are adsorbed by the membrane and affect their permeability. Murray notes that Shiga bacilli after extraction with ether cannot be burst by plasmolytic methods—an indication of increased permeability to electrolytes after removal of the fats.

We must note that membranes are also permeable to certain electrolytes in addition to the lipoids, which are clearly important as affecting their permeability. The experiments of A. Fischer (1895) (see also Osterhout, 1911) showed that plasmolysis occurred in the chlorides of the alkali metals and alkaline earths. This effect vanished in from 20 to 30 minutes, which was taken as an indication that the membranes were permeable although but slowly to these salts. Murray (private communication) found that the plasmolysis of *Meningococcus* is complete in 4 hours at 37° C. in salt solutions of 7·5 to 9·0 per cent. in strength. Salt then enters, and isotonicity is attained in some 30 hours or more. Blood corpuscles were shown likewise to be permeable, but slowly, to strong salt solutions. Whilst the viability of the organisms are not greatly affected by such treatment it is not certain that the membrane in its normal condition is permeable, for this transient osmosis can be effected by membranes breaking or coagulating under the influence of the electrolytes, and undergoing subsequent repair. In Ringer's solution the

plasmolysis of *Meningococcus* and *B. coli* remains constant. In yeast cells also but a small permeability is noted for salts, although alcohol passes readily through (Shearer, 1919; Paine, 1912). Meyerhof (1916) noted the permeability of *Nitromonas* for ammonia and the amines, but not for ammonium salts. Osmic acid, mercuric chloride and 25 per cent. alcohol were found by A. Fischer to penetrate cholera and typhoid organisms but slowly, whilst lactic acid and glycerin passed in rapidly. It seems probable from the evidence available that the lipoid-soluble materials, including the non-dissociated molecules of the fatty acids, pass freely, but the cations $\overset{+}{Ca}$, $\overset{+}{Na}$, $\overset{+}{K}$, as well as iron, magnesium and zinc occasionally found in small quantities inside cells cannot pass at all readily in the ionic form, but only in complex combination or through membrane material which has been temporarily modified. On the other hand, to the ions $\overset{+}{H}$, HCO'_3 and Cl' the membranes are apparently permeable as in the case of the blood-cell membranes. The $\overset{+}{NH_4}$ ion probably enters in the form of NH_3 or NH_4OH. We may note also that other phase boundaries exist in cells, such as between the gel-like endoplasm and the more sol-like contents of vacuoles in cells forming such vacuoles, and again in those portions of all cells capable of differentiation, such as the nucleus.

On altering the tonicity of the environment, e.g. by dilution, compensation of the turgor of the endoplasm can occur. Some organisms, e.g. those in sea water, can suffer great changes in environment (0·2 to 16 per cent. NaCl) without serious harm, others are peculiarly sensitive to osmotic changes. In many species there appears to exist an optimum tonicity for growth (about 3 per cent. NaCl for *V. choleræ*). Internal compensation of the turgor pressure can be effected by various means in addition to the usual ingress or egress of water through the membrane. Cases of negative osmosis in cylindrical but not spherical bacteria have been recorded by A. Fischer, who noted that cholera vibrios immersed in a 2 per cent. solution after treatment with 0·75 per cent. NaCl underwent swelling instead of the anticipated plasmolysis or shrinking.

The Conditions of Osmotic Equilibrium.

The extremely slow diffusibility or apparent non-diffusibility of the cations, with the possible exception of the hydrogen ion, through the membrane, draws attention to the importance of the proteins certainly in the endoplasm and probably in the ectoplasm in regulating the osmotic pressure. To these the membrane is impermeable, as shown by dialysis, and we thus have inside the cell a mixture of non-diffusible proteins capable of forming salts and diffusible electrolytes. As the assimilable foodstuffs of many bacteria are derived from proteins, it is not readily seen how they pass through the membrane; it is possible that they do not pass in as such, but are consumed by enzymes present in the membrane or degraded to simpler compounds before penetration.

Donnan membrane equilibrium. The conditions of equilibrium under these conditions were first laid down by Donnan. We may consider a simple case in which a membrane permeable to hydrogen and chlorine ions separates a hydrochloric acid solution from one containing a protein in the form of its ionized hydrochloride $\overset{+}{P}\,\overset{'}{Cl}$, the cation of which cannot pass through the cell membrane; the final distribution of ions on each side of the membrane can be depicted as

$$\begin{array}{c|c} \overset{+}{H}_1 & \overset{+}{H}_2 \\ \overset{'}{Cl}_1 & \overset{'}{Cl}_2 \\ \overset{+}{P}_1 & \end{array}$$

where the subscripts refer to the solutions. Donnan showed that the equilibrium distribution of ions in dilute solution was such that

$$\overset{+}{H}_1/\overset{+}{H}_2 = \overset{'}{Cl}_2/\overset{'}{Cl}_1$$

For strong solutions the ionic concentrations must be replaced by the thermodynamic concentrations or activities, and we obtain,

$$f_1\overset{+}{H}_1/f_2\overset{+}{H}_2 = f_2\overset{'}{Cl}_2/f_1\overset{'}{Cl}_1$$

where f_1, f_2 represent the activity coefficients.

As a general expression for a diffusible ions of various valencies we obtain

$$\frac{f_1 \overset{+}{M}_1 \overset{+}{M}_1}{f_2 \overset{+}{M}_2 \overset{+}{M}_2} = \frac{f_2 \overset{\cdot}{A}_2 \overset{\cdot}{A}_2}{f_1 \overset{\cdot}{A}_1 \overset{\cdot}{A}_1} = \frac{\sqrt{f_1 \overset{++}{M}_1 \overset{++}{M}_1}}{\sqrt{f_2 \overset{++}{M}_2 \overset{++}{M}_2}} = \frac{\sqrt[3]{f_2 \overset{\cdots}{A}_2 \overset{\cdots}{A}_2}}{\sqrt[3]{f_1 \overset{\cdots}{A}_1 \overset{\cdots}{A}_1}}$$

That is, the relative decrease in osmotic pressure between solutions separated by a semi-permeable membrane is the same for all univalent ions, the relative osmotic pressure of the cations being the reciprocal of the anions, and the relative decrease in osmotic pressure for an n-valent ion is the nth root of that for a monovalent ion.

The marked effect of a non-diffusible ionizable constituent on the membrane equilibrium is made clear from the following examples. Taking congo red as a univalent non-diffusible electrolyte in the form of its salt $\overset{+}{Na}\,\overset{'}{R}$, its effect on the distribution of sodium chloride on the two sides of a membrane is shown in Table II.

TABLE II.

Initial Ratio of NaCl to NaR on one side of the membrane. Water on the other.	*Percentage of NaCl passed when equilibrium is established.*
NaCl only	50
10 : 1	49·7
1 : 1	33
0·1 : 1	8·3
0·01 : 1	1·0

The experimental values of Donnan have been extended for this system, replacing concentrations by thermodynamic concentrations, by Azuma and Kameyama (1925). Donnan and Allmand (1914) studied the system

$$\begin{matrix} K_4Fe(CN)_6 \\ KCl \end{matrix} \;\Big|\Big|\; \begin{matrix} KCl \\ \\ Cu_2Fe(CN)_6 \end{matrix}$$

with the object of determining whether $(\overset{+}{K}_1)\ (\overset{'}{Cl}_1) = (\overset{+}{K}_2)\ (\overset{'}{Cl}_2)$ in accordance with the theory. In Table III are given some of their results for the ratio $(\overset{+}{K}_1)\ (\overset{'}{Cl}_1) \Big/ (\overset{+}{K}_2)\ (\overset{'}{Cl}_2) = R = 1$ according to theory.

TABLE III.

Concentration of $K_4Fe(CN)_6$ about 0·01 N.

KCl inside.	*KCl outside.*	*R.*
0·00085	0·00065	1·28
0·00085	0·00065	1·07
0·00085	0·00065	1·04
0·00085	0·00065	1·00
0·00085	0·00065	0·99
0·0423	0·0423	0·98

Kameyama has shown that, on insertion of the thermodynamic concentrations in the above system, the principle of ionic strengths is valid for the distribution equilibrium. With potassium iodide discrepancies were observed.

A further consequence of the Donnan theory of unequal distribution of diffusible ions on either side of a semi-permeable membrane when a non-diffusible ion is present on one side is that, when equilibrium is reached, there will exist a membrane potential difference.

Taking the system

$$\begin{matrix} \overset{+}{H}_1 \\ \overset{'}{Cl}_1 \\ \overset{+}{P}_1 \end{matrix} \;\Big|\; \begin{matrix} \overset{+}{H}_2 \\ \overset{'}{Cl}_2 \end{matrix}$$

As before $\overset{+}{H}_1 / \overset{+}{H}_2 = \overset{'}{Cl}_2 / \overset{'}{Cl}_1 = \lambda$

and by equating electrical and osmotic work we obtain for the membrane potential E_m

$$E_m = \frac{RT}{F} \log \lambda.$$

Hence by measuring the membrane potential and comparing it with the value calculated from the ionic concentrations a further confirmation of the theory may be sought. Donnan and Green (1914), working with potassium ferrocyanide solutions separated by a copper ferrocyanide and parchment membranes, established the existence of this anticipated

membrane potential. If E_m and E_p be the potential differences when ferrocyanide and parchment membranes respectively are employed then

$$E_m = (E_m - E_p) + E_p.$$

Equating E_p to the diffusion potential E_D, this latter being calculated from the expression

$$E_D = \frac{RT}{F}\left(u - \frac{v}{4}\right) \log \frac{f_1 C_1}{f_2 C_2},$$

the value E_m can be determined. The errors were somewhat large as uncertainty is introduced both in the assumption of the equality of $E_p = E_D$, and in evaluation of f_1 and f_2 for the equivalent ion.

Table IV shows the percentage error obtained.

TABLE IV.

Solution strength in normality.	*Percentage error.*
1 : 0·1	28·9
1 : 0·02	23·6
1 : 0·01	23·3
0·1 : 0·05	2
0·1 : 0·04	3
0·1 : 0·01	8·1
0·1 : 0·005	14·3

In the cases just considered the conditions of equilibrium of an ionizable protein salt mixture within the cell were established. Similar conditions will obtain if the non-diffusible constituents within the cell are not ionizable but can adsorb ions. The colloids in general do not adsorb cations and anions equally, but one ion is always adsorbed in excess of the other over appropriate ranges of concentration. The excess cations or anions adsorbed by the colloid within the cell are fixed, and are therefore rendered non-diffusible. Thus the anion P′ was considered to be non-diffusible in the case just considered; we may now regard P Cl, representing a protein complex P with an adsorbed Cl′, as the complex non-diffusible ion. Donnan's condition of equilibrium can likewise be applied to this case. The adsorption of ions by colloidal solutions of chromium hydroxide (Bjerrum, 1924), sulphur (Rinde, 1926), and silicic acid (Ganguly and Krishnamurti, 1928) have been examined and shown to fulfil these conditions.

Whilst Donnan's theory of membrane equilibrium has been shown to hold in the case of a number of non-diffusible colloidal ions, as well as of non-diffusible ion-adsorbing colloids, its general applicability to solutions containing proteins is still a matter of dispute; whilst accepted by Procter (1914) and Loeb (1924), it has been called in question by Robertson. On assumption of the validity of the Donnan equilibrium for proteins we may cite a few examples from Loeb's experiments. Dilute solutions of isoelectric proteins are suspended within a collodion sac. To the solutions varying amounts of acid are added and after equilibrium

is established (some 18 hours) the membrane potentials are determined. At the same time the hydrogen-ion concentrations of the inner and outer solutions are determined. If the equilibrium be in accord with Donnan's theory then we obtain

$$\begin{array}{c|c} \overset{+}{z}\,\overset{+}{p} & \overset{+}{\mathrm{H}}\,x \\ y\,\overset{+}{\mathrm{H}} & \overset{-}{\mathrm{Cl}}\,x \\ (y+z)\,\overset{-}{\mathrm{Cl}} & \end{array}$$

$$\mathrm{E}_m = \frac{\mathrm{RT}}{n\mathrm{F}} \log \frac{x}{y} = \frac{\mathrm{RT}}{n\mathrm{F}} \log \frac{y+z}{y} = \frac{\mathrm{RT}}{n\mathrm{F}} \log \left(1 + \frac{z}{y}\right).$$

The ratio $\frac{z}{y}$ may be varied by changing (1) the pH of the solution or (2) the protein concentration.

In Table V are given the data for gelatin and hydrochloric acid.

TABLE V.

100 c.cm. of 1 per cent. isoelectric gelatin.

c.cm. 0·1 N HCl added	1	2	4	6	8	10	12·5	15	20	30	40	50
Hydrogen electrode PD in millivolts	24·7	36	34·5	34·5	27	25·8	18·8	14	7·6	5·9	4·1	2·3
Membrane PD in millivolts ..	24·0	32	33	32·5	26	24·5	16·5	11·2	6·4	4·8	3·7	2·1

Dilute solutions of iso-electric casein were brought to a common hydrogen-ion activity of pH = 2·5 by the addition of hydrogen chloride, and inserted in a collodion bag, which was then immersed in hydrogen chloride solution of pH = 2·3. After 18 hours the membrane potentials were determined directly and also calculated from the pH of the solutions in equilibrium.

TABLE VI.

Casein chloride per cent. ..	4	3	2	1	0·5	0·25
Membrane P.D. millivolts ..	20·0	18·0	15·0	10·8	7·2	3·1
P.D. calculated from hydrogen electrode measurements.	21·5	19·2	16·2	11·2	5·9	4·1

It may be observed that the degree of solvation of a protein varies with its stage of ionization, thus rendering the system decidedly more complex than is the case with suspensions of lyophobic systems.

Osmotic Regulation.

We have noted that different micro-organisms respond at different rates to changes in their environment. There are a number of physico-chemical processes which can act as regulating mechanisms inside the bacterial cell; no one of these has been investigated in detail, but the following observations may be made:

(*a*) Alteration in membrane permeability to the solvent. Immersion in 1 per cent. peptone will stop osmotic swelling; this may be due in

part to strengthening of the cell wall, but it is more probable that the permeability of the membrane is altered by adsorption of capillary-active materials from the peptone.

(*b*) Alteration in selective permeability to solutes. We have noted that lipoid-permeable material can pass freely into the cell ; of the ions $\overset{+}{H}$ and Cl′ appear to be those most readily passed. In discussing relative osmosis we noted that the direction of osmosis was dependent on the adsorption, or electro-kinetic, as well as on the diffusion potentials. Transition from a normal to the abnorml osmosis by alteration in the concentration of electrolyte has been observed (Loeb, 1920).

(*c*) Sudden changes in the concentration of ions outside the cell may be compensated for inside the cell in two ways ; firstly by the buffer action of the salts and proteins present, and secondly by alteration in the rates of the metabolic and growth processes. The buffering powers of the contents of cells appear to be relatively great, each species tending to maintain a definite hydrogen-ion activity for its contents. The equilibriating mechanism may be expressed by means of the equation

$$\overset{+}{Na}P' + H_2CO_3 \rightleftharpoons \overset{+}{Na}HCO'_3 + HP$$

It will be observed that there is an increase in osmotically-active ions (HCO_3) within the cell as a result of entrance of carbonic acid, and swelling of the cell contents should occur.

Osmotic Equilibrium in Cells.

In the previous sections we have discussed the conditions of osmotic equilibrium and the possible modes of osmotic regulation. The experimental verification of the views has naturally been effected with artificial and macroscopic membranes. The extension of these concepts to living conditions was first made by Warburg (1922) in a study of the distribution of ions between blood corpuscles and serum. Whilst similar investigations on bacteria have not yet been carried out, the importance of the subject merits attention being drawn to the relevant facts concerning the distribution of electrolytes between blood corpuscles and serum which have been discovered since 1868 ; these comprise the following : (1) In equilibrium the blood corpuscles contain about half as much bicarbonate and chloride ions as the serum ; (2) the serum absorbs three times more carbon dioxide if blood corpuscles are present than if absent ; (3) saturation with carbon dioxide causes the blood corpuscles to swell ; (4) the corpuscle contents are more acid than the serum, although the membrane is permeable to hydrogen ions ; (5) the corpuscles of human blood are impermeable to the potassium ions in the corpuscles and to the sodium ions in the serum ; (6) oxyhæmoglobin is a stronger acid than hæmoglobin ; (7) the osmotic pressures inside and outside the corpuscles are equal ; (8) all the non-protein ions normally present are univalent : the ions Na, K, Cl′, HCO'_3

comprise 95 per cent. of the molecular concentration of blood electrolytes ; (9) hæmoglobin has a much greater base-binding power and buffer value than any protein in the serum.

These facts may be accounted for both qualitatively and in an approximate manner quantitatively by the following assumptions, cited by Van Slyke (1926).

1. The distribution of ions is governed by the Donnan membrane equilibrium. Denoting by subscripts s and c the concentrations in the serum and blood corpuscle respectively we obtain

$$\frac{\acute{A}_c}{\acute{A}_s} = \frac{\overset{+}{M}_s}{\overset{+}{M}_c} = D$$

2. Both corpuscle contents and serum are electrically neutral or (*a*) $\overset{+}{M}_s = \acute{A}_s$; (*b*) $\overset{+}{M}_c = \acute{A}_c + \acute{Hb}_c$ where $\acute{Hb}$ is the ionized hæmoglobin ion.

3. The total osmotic concentrations in the corpuscles and serum are equal.

$$\overset{+}{M}_c + \acute{A}_c = \overset{+}{M}_s + \acute{A}_s$$

This neglects the small contribution to the osmotic pressure of the ionized hæmoglobin.

Warburg (1922, p. 316) considered that the solutions were, in fact, not ideal, and that both cell and serum media were heterogeneous. If the volume of the disperse phase in the blood corpuscles be q_c, Warburg showed that

$$D' = \frac{\dfrac{\acute{Cl}_c}{\acute{Cl}'_s}}{\dfrac{\acute{HCO}_{3c}}{\acute{HCO}_{3s}}} = \frac{100/\{100 - q_c\,(1 - d\,HCO'_3)}{100/\{100 - q_c\,(1 - d\,\acute{Cl})}$$

where d HCO'_3, d Cl' are the respective distributions between protein and water phases in the blood corpuscles.

Experimentally q_c was found to be 40 per cent. at pH 6·5, and 34·5 per cent. at pH 7·9, and d (HCO'_3) to be 0·5. Assuming d Cl' to be zero, D′ is found to be 0·75 at pH 6·5, and 0·79 at pH 7·9.

Van Slyke and others (1923) on the other hand consider only the apparent bulk concentrations, but correct the osmotic pressure both for the influence of the hæmoglobin as well as the base bound by the serum proteins, and calculate D from the base-binding power of the cell and serum proteins, obtaining the results given in Table VII with three different kinds of blood.

TABLE VII.

Blood Sample.	$\frac{Cl'_c}{Cl_s}$	$\frac{HCO'_{3c}}{HCO_{3s}}$	D
1	0·577	0·597	0·637
2	0·679	0·723	0·738
3	0·792	0·830	0·832
1	0·616	0·586	
2	0·722	0·693	
3	0·837	0·802	

The calculation of D from the base-binding power of the corpuscle protein can be made from the following equation,

$$D = 1 - \frac{H'b}{2\overset{+}{M}_c - H'b}$$

where $\overset{+}{M}_c$ is the cation concentration in the cell and $H'b$ the cell protein anion. More complicated equations are obtained when the osmotic pressure of the hæmoglobin and the content of protein other than hæmoglobin are considered, and the original literature should be consulted.

Adsorption.

We have so far considered the bacterial cell wall as in the light of a semi-permeable membrane. In addition it presents other features of importance ; we note that in the case of bacteria the ratio of surface to volume is large, the specific surface of a coccus of 0·8μ diameter is 17·5 square metres per c.cm. of closely packed organisms. This large specific surface is not inert but in common with other surfaces it possesses the power of adsorption of materials from solution. Materials which lower the interfacial surface energy will be readily adsorbed from solution in amount determined by Gibbs law, which may be expressed in the following exact form :

$$\Gamma = \frac{-d\sigma}{d\mu}$$

where Γ is the excess adsorbed on the surface, σ the interfacial surface tension and μ the chemical potential of the capillary active solute, which is related to the activity and concentration in bulk solution by the expression $\mu = \mu_o + RT \log f_c$, or for dilute solutions $\mu = \mu_o + RT \log c$. The adsorption of capillary-active materials from culture media during multiplication of organisms results in a rise in the surface tension of the medium. As the organisms die, these, and possibly the other materials, are liberated, and the surface tension subsequently falls. The lipoids appear to be the important capillary-active materials concerned. Not only does the membrane permeability vary in different species of organism, but the variation in membrane composition is likewise reflected in

adsorption, thus whilst most bacteria suspended in water containing a trace of salt are electro-negatively charged, the trypanosomes are positively charged. The adsorption of water-soluble materials affecting adhesion to filter papers and infusorial earth is greater for Gram-positive organisms than for Gram-negative ones (Eisenberg, 1918).

Membranes, as we have seen in certain cases, can be burst by increasing the osmotic pressure of the cell contents, but owing to its composite structure it is also possible to disintegrate the membrane by other treatment. The term peptization is applied to the disintegration to colloidal dimensions of complexes in solution, and as Bancroft has pointed out, peptization is effected by adsorption of a capillary-active material on the outer surface, so as to effect a marked difference in surface tension between the two sides of the layer to be disintegrated. Such dispersion of cell walls can be effected not only by complex bodies formed within the bacteria (autolysis), but also by simpler reagents. It is found, for example, that Gram-positive membranes are readily peptized by fatty acids, Gram-negative ones by alkalis, whilst many membranes are peptized by lipoids and soaps, e.g. sodium taurocholate will disintegrate pneumococci, and lecithin and cobra venom will disintegrate typhoid bacilli.

It may be noted that the factor of adsorption is important in the action of germicides, thus the antiseptic action of mercury salts on yeast cells runs parallel with their adsorption, and an enhanced germicidal activity can be imparted to many substances, e.g. silver and mercury, by incorporating in the molecule a lipoid-soluble group. An interesting case of such action is that examined by Neergaard, who showed that the critical concentration of silver ions for inhibition of growth of *B. coli* in water was $1 \cdot 2\ 10^{-7}$; in broth the inhibitory concentration was only $1 \cdot 2\ 10^{-9}$, and at the same time it was shown by potentiometric measurements that the silver ions had reacted with some constituent in the broth to form a silver complex.

Since no direct method is as yet available for the actual determination of the interfacial surface tension between bacteria and the medium, we have to rely upon indirect evidence for support of the hypothesis. That an adsorption does actually take place, at any rate under certain specified conditions, is evident from the work of Küster and Bojakowsky (1912), who showed that the partition of phenol between water and anthrax spores followed the general empiric adsorption isotherm of Freundlich, $x = ac^{\frac{1}{n}}$, where x is the amount adsorbed per gm. of material, c the bulk concentration, a and n constants. Although it is not possible to measure the bacterium/medium interfacial tension, yet the measurement of the medium/air interfacial tension is a comparatively simple operation. If the assumption be made that most substances which affect the liquid/air interface will affect the membrane/liquid interface in a similar manner, an assumption for which there is much evidence from chemical data, but which is by no means universally true, then it follows that many

substances which depress the liquid/air interface will be strongly adsorbed by the micro-organisms, and affect either favourably or unfavourably their growth rate. This relationship between the lowering of the surface tension and germicidal activity is well exemplified in the somewhat insoluble hydroxyl derivatives.

In order of decreasing molar germicidal activities we find thymol, camphor, menthol, the cresols, and lastly phenol. These substances all depress the surface tension of water, thymol exhibiting a very marked effect, phenol being the least effective, the others lying between in the order given. Amongst the polyhydric benzene substitution products we again find this correlation between the surface tension depressant action and germicidal activity; for both these properties we find phenol > resorcinol > quinol > phloroglucinol > pyrogallol.

A remarkable correlation is likewise found in the nitrophenols and nitrobenzaldehydes. In the former the *p*-substituted products, in the latter the *o*-substituted products exert the greater molar germicidal activity; their action on the surface tension of water lies in this order. Brown and Tinker (1915) found a direct relationship between the adsorption of phenol by barley seeds and the surface tension. More recently (Reichel, 1914; Miller, 1920; Laird, 1920) it has been shown that the germicidal activity of phenol is raised by the addition of sodium chloride to the solution. This elevation is accompanied by an increase in the fugacity* of the phenol as measured by partition methods (cf. G. N. Lewis, 1901 and 1907). Since sodium chloride elevates the surface tension of water its surface concentration must be less than its bulk concentration, the surface concentration of phenol must consequently increase on the addition of salts. The germicidal activity of acids in the presence of neutral salts may be interpreted in a similar manner (Krönig and Paul, 1897; Kahlenberg and True, 1876; True, 1900; Haas, 1916; Norton, 1916). The inhibiting materials produced during bacterial growth may be soaps, fatty acids and higher aliphatic alcohols. Minute quantities of these materials affect the growth rate to a remarkable degree. Thus 0·02 per cent. octyl alcohol (Windisch and Dietrich, 1920) practically inhibits the growth of yeast. (See also Zoller and Clark, 1921, and J. H. Wright, 1917.) In many micro-organisms, especially in the yeasts, growth is accompanied by formation of fat, which can undergo gradual hydrolysis or saponification in alkaline media. The variability in ' lemco ' broth is generally ascribed to the presence of small traces of soaps formed during the process of manufacture. The saturated hydrocarbons possessing a high lipoid/water partition coefficient possess no germicidal activity, whilst the amount of chemical action between the hydrocarbon and water as estimated by the magnitude of the independent and interfacial surface tensions is likewise inconsiderable.

* The fugacity may be regarded as the thermodynamic or effective concentration, in contrast to the actual concentration required to impart the observed colligative properties to the solution.

Attempts have been made by Larson, Cantwell and Hartzell (1919) and Larson (1922), and others to determine the influence of the surface tension of the culture medium on the growth of bacteria, and to grade nutrient broth and peptone waters by such methods.

It is worthy of note that narcotics sensitize proteins to precipitation by electrolytes (Meyerhof, 1918), and thus germicidal action can be effected without the presence of a lipoid-soluble group, as postulated by the original theory of Hamburger. The surface tension of the medium frequently modifies the nature of growth of micro-organisms ; thus the addition of a small quantity of sodium ricinoleate to broth so as to effect a lowering of the surface tension below 40 dynes per cm. will prevent the formation of pellicle in *B. subtilis* (Larson and others, 1919 ; Green, 1921). According to Larson, all pellicle-forming bacteria are comparatively rich in acetone-ether-soluble fat-like substances, and bacteria grown for several generations in media containing an unfermentable carbohydrate or glycerol will develop a pellicle, and at the same time the acetone-ether-soluble constituents of the culture will rise. Substances strongly adsorbed may act as we have seen as germicides or effect marked changes not only on the nature of the growth, but also on the composition of the cells of the species. In addition, foodstuffs may be powerfully adsorbed, thus capillary-active foodstuffs when present form an ideal culture medium. The bile-salts are representative of such, in that they facilitate the growth of *B. coli*, but inhibit that of the cocci.

Chemical Constitution and Adsorption.

For ionized substances the sign and magnitude of the electrical charge are important factors, but not the only ones, the influence of the chemical nature of adsorbed ions being frequently marked, such as fluoride and oxalate ions reacting with intracellular calcium ions, whilst the anions of the lower fatty acids are somewhat lipoid-soluble. The adsorption of organic substances from solutions by bacteria is to be ascribed to the presence of certain groups in the molecules. The reverse of this, i.e. the non-adsorption in the absence of these certain groups has been clearly demonstrated by the work on plasmolysis of plant cells by de Vries (1884) and by that of Gryns (1896), on blood corpuscles, as well as by the later work of Overton and Lowe on plasmolysis through membranes, which indicated that membrane permeability for organic compounds was associated with the absence of certain active groupings. These active groupings in organic molecules are either acid or basic, e.g. — OH, — COOH, — NH_2, although unsaturated groupings such as the double bond and >CO and — CHO are not without influence. Evidently the adsorption is chemical in nature, a hypothesis utilized by Ehrlich in his work on the spirillocides and by Emil Fischer on the fermentation of the various sugars. The relationship between chemical constitution and surface tension clearly emphasizes the chemical nature of surface tension.

The surface film may be regarded as consisting of a unimolecular layer of orientated molecules attached to the water surface by their polar groups, apparently identical with the ' active ' groups of Hamburger and Lowe ; the non-polar portion, or the lipoid-soluble part of the molecule resists, as far as possible, immersion in the polar medium. No great extension of this hypothesis is necessary to include adsorption by micro-organisms. Lipoid-solubility of part of the molecule adsorbed is desirable so that the molecule may attach itself to the lipoid constituent of the cell, but it would appear that the metabolism of the organism is not interfered with by this process, as shown by the feeble action of the saturated paraffins. If, however, the molecule possesses active or polar groups, e.g. $-OH$, $-NH_2$, $-COOH$, these react with similar groups, but of opposite kind, present on the membrane of the micro-organism, and disturbance of growth occurs.

We must, therefore, postulate on the cell membrane or within its pores, at any rate in positions easy of access to the circumambient liquid, the existence of active or polar groups—acceptors, which can react with any molecule present likewise possessing active groups. It has been noted that these polar groups on the cell are either acidic or basic in nature. It would appear that many, if not all, micro-organisms possess both types of groups, e.g. both $-NH_2$ and $-COOH$, i.e. can react with both acidic and basic substances in solution, but the power of reacting varies for each bacterial species ; *B. coli*, for example, will react to both acid and alkaline groups, but is less resistant to basic germicides, its acid groups being predominant either in number or reactivity. *Staphylococcus* conversely is more sensitive to acid germicides. It is interesting to note that the former class of organisms are Gram-negative and the latter Gram-positive (Traube, 1919 ; Traube and Somogyi, 1921).

Selective Action.

The membrane surface possesses a definite structure, and its surface molecules are consequently definitely orientated relatively one to another. The presence of both acid and alkaline groups on the membrane has been demonstrated. Hence to each molecule building up the membrane surface, possibly a complex polypeptide, we must ascribe one or more such active groups or acceptors to which the material undergoing absorption is attached. To a submicroscopic observer the adsorbing surface will consequently no longer appear uniform, but will appear to consist of active groups both acid and basic, e.g. $-NH_2$, $-COOH$, $-OH$, distributed over it in a perfectly regular manner, giving the general appearance of a structure over a relatively wide area. It has been shown that union with either the acid or basic acceptors leads to a general classification of germicides, in some cases coincident with the division observed in Gram staining. Evidently this feeble exhibition of selective action of germicides dependent on the unipoint contact is greatly

augmented if the possibility of multipoint contact is envisaged. If the germicide possesses more than one active group, e.g. two $-NH_2$ groups in each molecule, these can evidently combine with two $-COOH$ acceptor groups on the micro-organism provided that the distance apart between the two groups on the germicide is comparable to the distance apart of the acceptors on the micro-organism. Since the bacteria will vary among themselves both in number, nature and spacing of the acceptor groups, a very selective action is to be expected, provided the suitable reacting germicide can be found or synthesized. In favour of this hypothesis may be mentioned the facts that the dyestuffs possessing more than one active grouping are more selective in their action than the ions or simple organic compounds, e.g. methylene blue (Binger, 1919 ; Fraser, 1920), diphenyl and triphenyl-methane dyes (Stilling, 1871, 1904, 1906 ; Crossley, 1918), rosanilines (Crossley, 1918), flavine (Browning, Gulbransen and Thornton, 1917), and trypan red (Ehrlich, 1907).

An interesting series of observations by Nirenstein (1920) on the staining of *Paramecium* reveals the differences to be anticipated, if the membrane is thus viewed as containing both lipoids and acid and basic groups and not only pure lipoid as postulated by the hypothesis of Overton. Nirenstein compared the staining power of a number of dyes with their partition coefficients between water and (*a*) olive oil, (*b*) olive oil containing oleic acid, (*c*) olive oil containing diamylamine, (*d*) olive oil containing oleic acid and diamylamine. The correlation between staining power of both acid and basic dyes, and the partition coefficients was most complete for (*d*), good for (*c*), for acid dyes only, and for (*b*) with basic dyes.

The combination of toxins and antitoxins and the reactions between the various sugars and their particular enzymes are simply interpreted on this hypothesis of multipoint contact. The hypothesis leads to an interesting speculation as to the possibility of enzyme synthesis by the insertion of the particular reactive groups at the proper distance apart in a hydrocarbon chain, by hydrolysis of the complex cell structure of the micro-organism which produces the enzyme, and by bridging over the gaps between the reactive groups on the membrane surface by chemical reaction. Evidently the old simile of key and lock can readily be restated in terms of complicated multipoint contact produced by intermolecular reaction.

In processes of agglutination it would appear that two factors have to be taken into consideration (for a résumé of the literature see Bogue's Colloidal Behaviour, Vol. I, p. 70). Bacteria may be agglutinated by many, especially multivalent, salts, or by the addition of egg-albumin or agglutinins. In general, precipitation or agglutination can only occur when the adsorption of electro-kinetic potentials of the bacteria is lowered to within $\pm$ *ca* 15 millivolts of their iso-electric points. Salts may effect this lowering by preferential adsorption of ions of sign opposite to that of the charged bacterium. In general both ions are adsorbed, with the result that the lyotropic series of the cations varies with the nature of the anions

employed. Both egg-albumin and precipitins may effect a lowering of the electro-kinetic potential, and thus cause precipitation, irrespective of the alkalinity or acidity of the medium, within wide limits. It is clear that egg-albumin and precipitins cannot be the actual precipitating agents, but they are adsorbed by the bacterium irrespective of the sign of their electric charge, and the bacterium so protected is now rendered sensitive to precipitation by electrolytes in the system.

The experiments of Neisser and Friedemann (1904), Buxton and Shaffer (1907), and Northrop and De Kruif (1922), indicate, however, that a lowering of the electro-kinetic potential to within $\pm$ 15 m.v. of the iso-electric point of the bacterium is not in itself a criterion of the conditions of agglutination, since uni- and bi-valent ions are not effective in agglutination, although they are selectively adsorbed strongly enough to effect a lowering of the electro-kinetic potential to within the necessary ranges. Agglutinin-sensitized bacteria, however, are readily precipitated under these conditions. A simple explanation of this remarkable result is forthcoming, if it be assumed that the protein carbohydrate complex is in the normal state covered with a thin film, possibly a unimolecular layer of lipoid. Agglutination appears to be a property which is not possessed by the lipoid-coated bacterium, but if agglutinins or albumin are adsorbed on to the lipoid surface, agglutination can take place. Again, highly charged polyvalent ions as well as those that readily coagulate proteins can penetrate the lipoid surface and affect the protein underneath, with the result that the lipoid layer disappears at these points, exposing the protein, which can now effect agglutination on contact with other bacteria.

Enzyme Action.

Two views are held as to the nature of the enzymes elaborated by micro-organisms. By some they are regarded as definite chemical compounds of high molecular weight, colloidal and lyophilic in nature, containing both nitrogenous compounds and carbohydrates, but not giving the usual protein reactions. The alternative view is one which has been suggested in the previous section ; namely, that whenever on the surface of the complex mosaic containing both protein and carbohydrate there appear a certain number of reactive groups, e.g. —CHO, —COOH and $—NH_2$, in a particular configuration, these form the ' enzyme ' surface, capable of causing reaction in adsorbed substances of suitable stereochemical configuration and containing likewise suitable and suitably spaced reactive groups. This hypothesis of a mosaic possesses certain advantages over the former view of definite chemical constitution, for it permits of the existence of a great number of ' enzymes ', each being merely a special grouping of active groups in the mosaic without involving the necessity of elaboration by the micro-organisms of a great variety of distinct and complex chemical compounds. Organisms such as *B. coli* possess the faculty of bringing about a number of ' enzyme ' reactions,

these can be affected by poisons such as ultraviolet light, chlorine water and toluene (Quastel and Wooldridge, 1927) and it is found that the various reactions can be differentiated in respect to their sensitivity to poisons. Here again it seems more reasonable to assume that the poisons are selectively adsorbed on different active patches of the complex mosaic of the cell membrane and intracellular surfaces, rather than that specific chemical combination occurs with enzymes of definite chemical constitution. In addition it may be noted that as long as the structure of the small portion of the mosaic which is responsible for the enzyme action under investigation, remains undisturbed, processes of purification in eliminating other portions of the mosaic may be employed. We should anticipate that such purification processes might destroy certain portions of the mosaic, which, possessing a different chemical structure, can catalyse other reactions without affecting one particular group.

The enzymes are strongly adsorbed by numerous substances such as charcoal, alumina, fats, cholesterol and calcium oleate. When adsorption is effected by an enzymic active group the enzyme is naturally rendered inactive, but when effected by a non-active group the activity is unaffected, thus invertin is unaffected in activity when adsorbed on alumina. Willstätter and others (1922) have shown that in many cases co-enzymes or inert adsorbing materials may accelerate the action between enzyme and reactant; thus calcium-oleate-albumin mixtures accelerate the hydrolysis of fats by lipase. This increase in activity on addition of the co-enzyme may be due to more effective dispersion of the enzyme, or of the active groups of the enzyme, due to adsorption by the co-enzyme, similar to the increase in activity noted in the dispersion of platinum on silica gel or gum arabic, or it may be a case of so-called promoter action frequently observed in catalytic processes, where many examples of augmentation in velocity of surface actions are to be observed when two catalysts A and B are replaced by a number of phase or possibly even molecular boundaries in a mixture A B. The reaction kinetics of enzyme reactions are most readily interpreted on the assumption that the reaction velocity at any time is proportional to the concentration of active enzyme-substrate-complex present. On this simple assumption the reaction velocity is given by the expression:

$$\frac{dx}{dt} = \mathrm{K}\,\frac{x\,\varepsilon}{x+k}$$

where x is the concentration of the substrate, ε that of the enzyme and K the dissociation-constant of the enzyme-substrate-complex. This simple formulation is complicated by the other factors. The products of reaction are frequently adsorbed, thus invertin adsorbs both cane sugar and fructose. If the product is strongly adsorbed it may displace the reactant from the surface of the enzyme, a process leading to the well-known Schütz's law

$$\frac{dx}{dt} = \frac{\mathrm{K}}{x} = \frac{\mathrm{K}^1}{\sqrt{t}} \text{ or } \frac{\mathrm{K}}{\mathrm{K}^1} = \frac{x}{\sqrt{t}}$$

We may cite as example, Herzog's data (Oppenheimer, 'Die Fermente und ihre Wirkungen', 4th edition) on the hydrolysis of ethyl-acetate by lipase.

TABLE VIII.

Time.	*Percentage Hydrolysed.*	$\frac{x}{\sqrt{t}}$
5	3·5	1·6
10	5·8	1·8
15	7·0	1·8
20	7·8	1·7
30	10·1	1·8
45·25	12·8	1·9
60	14·4	1·9

The enzymes are very sensitive to changes in hydrogen ion concentration, there being usually a well-defined optimum pH (Kuhn, 1922; Euler and others, 1923; Michaelis and Rothstein, 1920). We may either regard the enzyme as an amphoteric electrolyte in which the non-ionized portion is catalytically active or consider that the complex enzyme substrate is amphoteric, and only reactive in the ionized form.

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Bacterial Pigments.

BY HAMILTON MCCOMBIE.

Pigment-forming Bacteria.

The power of producing colouring-matter is very widespread in the domain of micro-organisms. The actual colours which occur vary very much according to the micro-organism. Thus *B. luteus* and *Micrococcus pyogenes aureus* produce a beautiful lemon-yellow pigment; among the numerous bacilli producing red pigments are *B. prodigiosus, Spirillum rubrum,* and pink yeast; there are numerous yellow-green, dark green or blue fluorescent bacilli; a blue dye mixed with a fluorescent substance is found in the case of *B. pyocyaneus* and the bacillus of blue milk (*B. cyanogenes*); *B. violaceus, B. janthinus, B. coeruleus* and *B. indigonacius* produce pigments varying from pale blue to violet.

In most cases the pigment does not diffuse into the surrounding medium during the life of the bacillus, but only after death. On the other hand, in some cases, the colouring matter is distributed through the medium, giving it a characteristic appearance. Several attempts have been made to utilize this behaviour as a basis for the classification of bacteria. Thus, Beijerinck (1891) suggested three classes of bacteria : (*a*) Chromophoric bacteria in which the pigment is contained in the body of the bacterial cell ; (*b*) parachromophoric bacteria in which the pigment is attached to the envelope ; (*c*) chromoparic bacteria in which the pigment is a true excrement.

In chromoparic micro-organisms, as in the chromophoric, the dye is always produced within the cell, but is quickly discharged. It may be discharged (e.g. in *B. pyocyaneus*) in the form of a leuco-body which becomes coloured by exposure to oxygen. Such deferred production of colour may also occur in indigo fermentation, and, to a certain extent at least, in the formation of pigments by bacterial action on certain aromatic substances.

Chemical Constitution of Bacterial Pigments.

Very little is known about the chemical constitution of bacterial pigments, because only in very few cases indeed have these substances been obtained in pure, homogeneous crystalline condition. Only a few of the pigments are soluble in water : the majority are insoluble in water but are soluble in alcohol, acetone, ether or chloroform. The isolation of the pigments is often rendered extremely difficult by the oxidations and reductions which they undergo. The green colours of certain bacteria have been identified with chlorophyll by some workers, but conclusive experiments on the subject are lacking. The red colouring-matter of the purple bacteria was isolated and studied by Ray Lankester (1873) who gave to this pigment, which may vary in colour from peach colour to intense violet, the name ' bacteriopurpurin '. It is insoluble in water, alcohol, chloroform, ammonia and acetic acid and has been studied by many subsequent workers, especially by Molisch (1907). From a spectroscopic study of bacteriopurpurin Molisch distinguishes two varieties of pure dyes, and he explains the varying spectra of the living cultures as due to admixture with a green dyestuff. It is considered by some writers that bacteriopurpurin is closely related to carrotene which occurs widely distributed in the plant and animal world. Prodigiosin, the colouring-matter from *B. prodigiosus* has been studied by Griffiths (1892) and Kruppt (1902), but it is certain that it has never been obtained pure. Pyocyanine, the pigment produced by *B. pyocyaneus* is easier to isolate than most pigments, because it is a basic substance and consequently can be extracted easily by dilute acids. It was first obtained by Fordos (1861) and was investigated later by Gessard (1882) and Ledderhose (1888). At a later stage the chemical constitution of pyocyanine was investigated by McCombie and Scarborough (1923) and by Wrede and

Strack (1924). From this work it seems likely that pyocyanine contains two phenazine neuclei united by peptide linkings. Finally mention should be made of cytochrome which has been studied extensively by Keilin (1925). This is an intracellular respiratory catalyst common to animals, bacteria, yeast and higher plants. It is easily oxidized by air and reduced by the normal cell and by chemical agents. Cytochrome probably contains three components, of which two have an iron-pyrrole nucleus similar to that of hæmoglobin.

Conditions for Pigment Formation.

Oxygen seems to be essential for the development of colour and consequently in stab cultures and in plate cultures only the surface colonies are found to be coloured. In certain cases, however, the colour is not developed on the surface but only inside the medium. Many bacteria produce pigments only on definite media ; thus the red bacillus produces its pigment only on potato, *B. pyocyaneus* produces the blue pigment only when peptone is present in the medium, the bacillus of green sputum produces its pigment only when albumin is present. Temperature is an important factor in pigment production and in general raising the temperature of growth of the organism gives rise to a decrease in colour formation. Light seems necessary for the development only of such pigments as are produced by the green and purple bacteria. In other cases pigment formation takes place best in the dark. Strong illumination generally destroys the colour.

The nature of the medium plays a large part and one which is understood but slightly. There are some bacteria which when grown in a liquid medium yield only traces of pigment, while on a solid medium they give considerable quantities. In certain cases also it has been established that definite mineral salts must be present in the medium if pigment formation is to take place : thus magnesium sulphate must be present in the medium in order to obtain colour formation in the case of *B. prodigiosus* and *B. pyocyaneus*. It is quite likely, however, that these particular salts favour pigmentation merely by favouring the growth of the organism. The reaction of the medium often has a marked effect on the colour of the pigment : thus the bacillus of blue milk on a neutral medium produces a gray pigment, on alkaline media, a red one, and on acid media, a blue.

All conditions which tend to diminish the activity of the organism reduce its pigment production. Successive cultivation of several generations often result in decreased colour formation, but, on the other hand, in certain cases, a careful choice of media can give rise to the production of more intense colours.

Physiological Role of Bacterial Pigments.

Little can be said about the physiological role played by the bacterial pigments. It is possible that, in certain conditions, they serve as a means of fixing oxygen. In any case their affinity for oxygen is most marked and generally the pigments are easily oxidized. According to Beijerinck

(1891), the dyestuff is of importance for nutrition in chromophoric micro-organisms only, while in the chromoparic and parachromophoric it is a useless secretion; but it is doubtful if that position can be maintained at the present day.

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CHAPTER IV. GROWTH AND REPRODUCTION OF BACTERIA.

By Arthur Slator, A. D. Gardner (University of Oxford) and H. G. Thornton (Rothamsted Experimental Station).

Growth and Reproduction.

By Arthur Slator.

Phases in the Growth of Bacteria.

Introduction.

The number of chemical reactions taking part in the development and growth of bacteria and other similar micro-organisms is very great. Certain constituents in the medium in which the culture grows undergo a complete chemical change, and some of these fermentations have been closely studied. The degradation, for instance, of glucose by *B. coli*, and the mechanism by which lactic acid, pyruvic acid, acetaldehyde, acetic acid and alcohol are successively formed have been worked out by Stephenson and Whetham (1924). The mechanism by which sugars are converted into alcohol and carbon dioxide by yeast has probably been more thoroughly investigated than that of any other fermentation. Accompanying these changes in the medium, the development of the micro-organism takes place. From comparatively simple substances present in the medium, the complex proteins, carbohydrates, cellulose-like substances, gums, fats, nucleic acid and other bodies are synthesized by the living bacteria and make up the cells of new organisms. The growth of the micro-organism is made up of a large number of these up-grade chemical changes, which of their very nature are more difficult to study and understand than the down-grade ones brought about in the medium in which the organism is growing. The two sets of reactions are doubtless connected or coupled together in some way, for otherwise it would be difficult to understand how the up-grade reactions could take place at all. The growth of the micro-organism is definitely bound up with the chemical changes taking place in the medium.

It is remarkable that in spite of the very complicated nature of bacterial growth, development and reproduction under favourable conditions take place regularly and in an orderly manner. Definite phases of growth can be observed, and rates can be measured with reasonable accuracy. If a small seeding of a culture of bacteria be introduced into a suitable nutrient medium, the organism takes some little time before rapid development starts, and the so-called lag-phase in growth is first observed. When this is over, the rate of development

is determined solely by the number of cells present, and the logarithmic period of growth takes place, where the number of cells increase logarithmically with the time.

Retarding influences then come into play and growth is arrested. Among the factors which cause this retardation, one of the most important is the accumulation of products of metabolism. Lack of food or of one particular food, which may have been used up by the organism in its growth, or have disappeared owing to fermentation by the growing organism, is also a potent factor in arresting growth. The cells, as they get older and as the medium changes, tend to pass into a resting stage and cease to multiply. Growth finally stops and later the number of cells tends to lessen, and death-rates come into consideration.

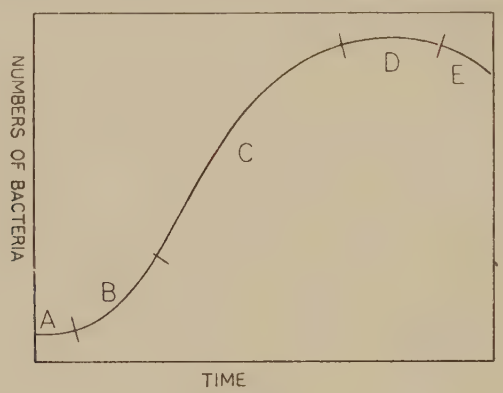

FIG. 1.

The periods of growth A, B, C, D and E, as shown in Fig. 1, can be conveniently considered under these different headings. A represents the lag-phase in growth, B the logarithmic period of growth, C the period of retarded growth, D the maximum growth, and E the death of the cells. The work on these different periods of growth has lately been summarized and discussed very thoroughly by Buchanan and Fulmer (1928). Whilst under favourable conditions all these stages of growth can be observed, under other conditions one or other of them may disappear. Thus, if actively growing cells be used for seeding purposes, the lag-phase is not observed. If the seeding be large, the retarding influences will accumulate so quickly that the logarithmic period does not occur, and a very large seeding may prevent growth entirely, the cells never getting over the lag-phase.

Logarithmic Period of Growth.

Under the simplest conditions of unrestricted growth, the rate depends only on the number of organisms present. The growth then follows the logarithmic law, the number of cells increasing geometrically with the time, according to the equation $\frac{dn}{dt} = K \cdot N$. The constant of growth (K), or the generation time (G.T.) remains the same over this period.

If the number of cells N increase to N + n in time t, then K is given by the equation $K = \frac{1}{t} ln \frac{N + n}{N}$, and the generation time is equal to $\frac{ln\, 2}{K}$. This period of growth has been observed in the case of many bacteria and of yeast cells.

J. E. Lane-Claypon (1909) measured rates of growth of *B. coli*, *B. typhosus*, *B. enteritidis* (Gaertner) in nutrient broth. The number of bacteria was estimated by ' plating ' and the influence of temperature was specially studied. The figures given in Table I are typical of the results obtained.

TABLE I.

B. coli growing at 42° C.

Time after Commencement of Experiment.	Number of Bacteria per 0·02 c.cm.	K.	G.T. (hours).
2 hours 0 minutes	87	—	—
3 ,, 40 ,,	2,876	0·91	0·33
4 ,, 50 ,,	36,675	0·93	0·32
6 ,, 10 ,,	739,200	0·95	0·32
	Average ..	0·93	0·32

Penfold and Norris (1913) estimated the constants of unrestricted growth of *B. typhosus* in peptone water. They found that rates of growth were proportional to the peptone concentration when this concentration was below 0·2 per cent. Slator (1913) described several different ways of measuring rates of growth of yeast over this period. The time-fermentation curve over this period is a logarithmic one, and the constant of the curve is the constant of growth. Measurements of rates of fermentation can, therefore, be used to obtain constants of growth. It was found by direct counting that a yeast growing in malt-wort showed a G.T. 2·95 hours at 20° C. In a fermentation experiment on the same yeast, a G.T. of 3·0 hours was obtained. Another method which gave a result in agreement with these two is as follows : Two experiments were made in which wort was seeded with small amounts of yeast in a known ratio. Fermentation took place in each case, but earlier in the case of the larger seeding than the smaller. The lag of the one fermentation behind the other gives the time for the amount of one seeding to grow to that of the other. The G.T. measured in this way was found to be 2·85 hours.

Yeast is a relatively large organism and lends itself to observations of growth under the microscope. In some experiments on a baker's yeast growing at 30° in malt-wort G.Ts. were measured successfully by timing

the appearance of buds. The G.T. is the time which elapses between a cell budding and the same cell budding a second time. Under favourable conditions both mother- and daughter-cell bud practically simultaneously and the G.T. is the time $t_2 - t_1$ as represented in Fig. 2.

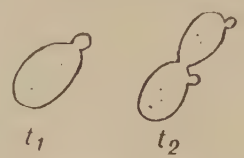

FIG. 2. Generation time $= t_2 - t_1$

The yeast in question showed by direct measurements a G.T. of 73 minutes. Twelve individual cells were watched under the microscope and gave values 78, 66, 76, 70, 79, 65, 81, 72, 72, 70, 72, 73 minutes, the average being 73 minutes. The G.T. of individual cells varied up to 8 minutes on each side of the average (Slator, 1918).

As an example of a nephelometric method of estimating rates of growth, the growth of *B. delbrücki* in malt-wort at 45° C. may be quoted (see p. 153).

Table II shows that this method can give good constants of growth.

TABLE II.

t hours	$\frac{N + n}{N}$	$0{\cdot}434\ k$
2·53	119	0·82
3·48	800	0·83
4·00	2,260	0·84
4·73	9,060	0·84
5·02	18,100	0·85
		0·84

$$\text{Generation time} = \frac{\log 2}{0{\cdot}84} = 0{\cdot}358 \text{ hour} = 21{\cdot}5 \text{ minutes.}$$

Lag-phase in Growth.

When a culture of a micro-organism is seeded in small amounts into a fresh nutrient medium in which it eventually grows at a logarithmic rate, there is initially a slower rate of growth, which has been termed the lag-phase in growth. This has been observed by many investigators who have measured rates of growth of bacteria and other micro-organisms. Penfold (1914) investigated the effect of various influences on the lag in the growth of *B. coli* in 1 per cent. peptone (Witte) + 0·5 per cent. salt. He found that the older the parent (within limits) the longer the lag. If the maximum rate of growth be interrupted by a short application of cold, growth recommences without lag. If the cold be long continued, lag will tend to reappear. Lowering of temperature lengthens this period of lag. Penfold suggests that the bacterial protoplasm is synthesized in steps,

and that time is required to attain optimum concentrations of essential intermediate bodies before the maximal rate is attained. A likely explanation of the lag-phase is given on these grounds. Ledingham and Penfold (1914) have submitted some of Penfold's results to mathematical analysis, and find that time and number of bacilli over this period are connected by an equation $X^n = K \log Y$ where n and K are constants, Y being the number of bacteria after the time X. A relationship between n and K was noted by Slator (1917), and the equation can be put in the form of $X^n K^n = \log Y^n$, the advantage being that K was constant for all the experiments.

Some estimations in the lag-phase of yeast-growth were made by Slator (1918), who watched the growth of the organism under the microscope. Old yeast cells remained quiescent for various times, and when they budded they grew at the maximum rate. In such cases the Ledingham-Penfold equation would not apply. Tait and Fletcher (1926) also made a study of the lag-phase in the growth of yeast cells, but were unable to offer a simple explanation of the changes which take place during the storage of yeast and which bring about lag in growth at the commencement of a fermentation. They found remarkable differences in the keeping qualities of pressed yeast which had been previously washed with water and pressed yeast obtained directly from its own beer. The washed yeast lasts longer, contains less dead cells and liquifies less rapidly than the unwashed yeast. They discuss the reasons of this difference and conclude that in unwashed yeast there is a steady accumulation of by-products which bring about weakening of the cells and premature death. In washed yeast where by-products have been largely removed and are not re-formed, the yeast in order to maintain life gradually depletes its reserves with consequent reduction in vitality. Both the washed and unwashed yeast show lag in growth when seeded into fresh wort. The unwashed yeast cells have first to expel the accumulated by-products and also absorb the food necessary for rebuilding their starved plasma. In the case of washed yeast the by-products may be less in amount and toxicity, but still the yeast has to rebuild its depleted plasma. The length of the lag-phase period must be some function of the extent of the cell disintegration.

Period of Retarded Growth.

The unrestricted growth of bacteria in a nutrient medium soon ceases, owing to the effect of retarding influences, such as scarcity of food or the production of substances toxic to growth. Growth proceeds with increasing difficulty. This period of retarded growth is, however, in some respects the most important of all, for even though growth is retarded the actual increase in numbers is great, and when the maximum crop is obtained, it is found that the majority of the cells have been produced during this period. Mathematical equations have been devised to cover this period of growth, but under ordinary conditions of bacterial development, several

retarding influences acting in unknown ways must come into consideration, making the matter too complicated to be represented generally by such equations.

The rate of unrestricted growth depends only on the number of bacteria present and is determined by the equation $\frac{db}{dt} = Kb$ where b is the number of bacteria at time t. Growth under these conditions is unlimited and such conditions last only a short time. Later retardation may take place owing to lack of food, and under the simplest conditions it can be assumed that the rate is proportional to the food supply. If a is the maximum count, the value $(a-b)$ is a measure of the food supply and the rate under these conditions is determined by the equation $\frac{db}{dt} = Kb\ (a-b)$, b being the number of bacteria, and $(a-b)$ a measure of the food supply.

This is the typical equation of an autocatalytic chemical change. The equation can be integrated and if b is plotted against t the characteristic sigmoid curve is obtained. Attempts have been made to apply this equation to bacterial growth. As an example may be quoted the growth of *B. dendroides*, a typical ' spreader ' growing on an agar plate with potassium nitrate and asparagin as food (Thornton, 1922). The area of the colony y was found by Lotka (1925) to be related to the time x according to the equation

$$y = \frac{0{\cdot}2524}{e^{-2{\cdot}128x} + 0{\cdot}005125}$$

The agreement between the observed area and the calculated value is good, as shown in Table III.

TABLE III.

Age of Colony. Days.	Area in sq. cm.	
	Observed.	Calculated.
0	0·24	0·25
1	2·78	2·03
2	13·53	13·08
3	36·30	37·05
4	47·50	47·39
5	49·40	49·02

In this case the factor retarding growth must be a simple one and there is evidence to show that the disappearance of the water film on the freshly poured agar-plate is this limiting factor.

Maximum Growth.

The factors which determine the maximum growth in a nutrient solution can be best described by first considering a simple example. If conditions are so arranged that one single factor retards and finally stops

growth, the maximum growth in the medium admits of calculation. Thus, if yeast cells were developing in a medium and the conditions were so arranged that the sugar in the medium, which is used up both by supplying food to the yeast cells and by fermentation of the sugar, finally failed and stopped growth, then the following considerations are of importance. During the logarithmic period of growth, the increase in the number of yeast cells N, during the time S gm. of sugar disappear, is connected with K, the constant of growth, and F, the fermentative activity, of the yeast, by the equation $N/S = K/F$. During any other period of growth $\frac{dN}{dS} = \frac{K}{F}$ holds good, and N is given by the equation $N = \int \frac{K}{F} dS + \text{constant}$. Yeast crops (from a small seeding) are, therefore, determined by the way this ratio K/F varies with different concentrations of sugar. If the ratio K/F is independent of the concentration of sugar (this is substantially correct), the growth under definite conditions is a constant. The yeast crop is the sum of the seeding and the growth, the latter being constant. Again, the ratio K/F varies little with temperature, and yeast crops over a certain short range in temperature are also constant. This general formula (Slator, 1921) is of considerable use in obtaining a mental picture of what is of importance in determining the maximum growth of an organism in a given medium. Under ordinary conditions of growth more than one factor plays a part in retarding and stopping development, and the maximum growths would not admit of exact calculation.

Death-Rate of Bacteria.

Bacteria under adverse conditions tend to die, and the factors which determine the death-rate have been studied, and are described in Chapter V. Under some circumstances death-rates are apparently logarithmic. Under others the relationship between time and the death of the cells is more complicated, as is shown to be the case by Fulmer and Buchanan for yeast cells.

Methods of Estimating Living and Non-Living Members of a Culture.

If the organism under consideration is not too small in size the number of cells can be estimated by use of a microscope and a hæmocytometer. A ruled instrument such that each square corresponds to 1/4,000 c.mm. is often employed. Another method is to use an eyepiece of the microscope with a diaphragm to adjust the size of the field. In such cases the number of cells in the whole field is counted. Some idea of the proportion of living and non-living cells can be obtained from the appearance of the cell, and help can be given by staining methods.

By the well-known method of ' plating ', the number of living cells in a culture can be counted. In some cases a proportion of the living cells

perish in the solid medium : in other cases the solid medium aids growth, and cells which would eventually die in the liquid medium grow in the same medium made solid by agar or gelatin.

Nephelometric methods are of considerable use under certain circumstances. Some long bacilli, such as *B. delbrücki*, show a silky appearance when the medium in which the organism is growing is gently agitated. This is due to the organism setting in the direction of the convection currents. A similar appearance is noticeable with a suspension of asbestos in water. A series of tubes containing asbestos in increasing amounts is prepared and a growth of bacteria in a liquid medium can be matched against one of the standards and a measure of the growth obtained. This method was used to estimate rates of growth of *B. delbrücki* in malt-wort (Slator, 1916).

Barium sulphate suspensions in water can be used to estimate concentrations of yeast cells. Peskett (1927) finds it possible to carry out such estimations when the yeast concentrations lie between 0·5 and 35 million cells per c.cm. A general method of measuring the relative turbidity or opacity of two fluids including that of bacterial emulsions is described by Dreyer and Gardner (1916). By centrifuging and measuring the amount of deposit the volume of bacteria present can be estimated. Weighing the amount of bacterial growth by filtering through a Pregl tube and weighing with a micro-balance is also possible (H. I. Coombs and M. Stephenson, 1926).

The amount of yeast-growth in a medium can be estimated by determining the loss of nitrogen in the medium after separating the yeast (H. T. Brown, 1909). Rates of fermentation under certain conditions give a measure of the quantity of yeast or bacteria present in the fermenting medium.

R. H. Hopkins (1927) discusses in some detail four methods of measuring yeast-increases in a wort (1) counting cells by the hæmocytometer method, (2) weighing the yeast, (3) centrifuging, (4) nitrogen assimilation. The advantages and limitations of each method are described.

Relationship of Growth to Various Factors.

Temperature.

Bacteria and other micro-organisms can grow only between a comparatively narrow range of temperatures, the minimum being just above 0° and the maximum about 55° C. Different species show growth at different ranges of temperature ; some grow most readily at a comparatively high temperature, while others prefer a lower temperature. By making a series of cultures at various temperatures, it is possible to determine that at which growth takes place most readily. This optimum temperature varies with the medium. If, for instance, there is in the medium a constituent which retards growth, the inhibiting effect will be most marked at high temperatures, and in such cases the optimum temperature is lowered. A more exact optimum temperature is obtained by measuring

generation times at various temperatures. In the case of *B. delbrücki* growing in malt-wort the following results were obtained (Slator, 1916) :

TABLE IV.

Temperature.	Constant of Growth.	Generation Time (minutes).
58·0 °C.	No growth	—
55·0 ,, ...	Slight growth	—
52·5 ,, ...	0·54	33½
50 ,, ...	1·00	18
45 ,, ...	0·84	21½
40 ,, ...	0·62	29
35 ,, ...	0·365	50
30 ,, ...	0·184	98
25 ,, ...	0·084	215
22 ,, ...	0·02	900
20 ,, ...	No growth	—

These results, which are also given in the accompanying curve (Fig. 3), show a maximum rate of growth at 50 to 51° C., and no growth above 58 or below 20° C. The temperature quotient of the rate of growth

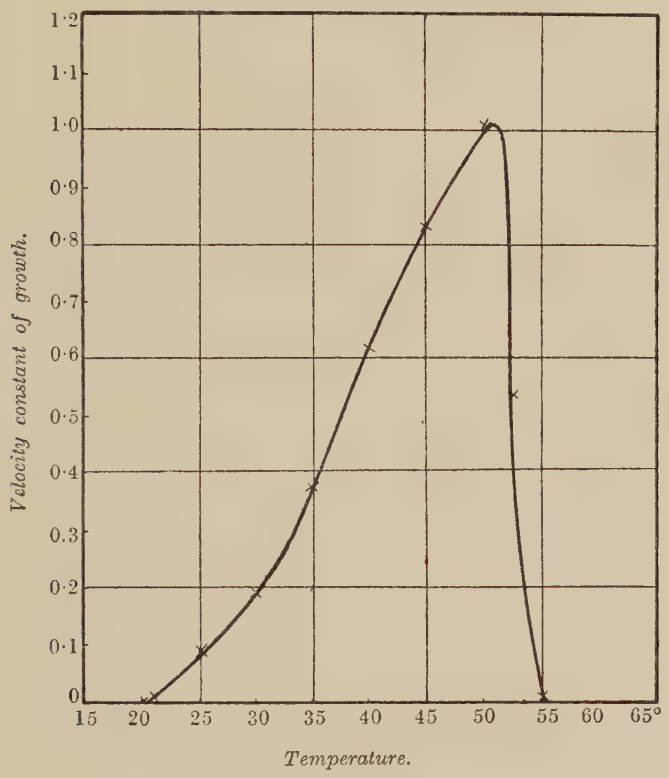

FIG. 3.

varies from a very large value at about 20° C. to 1 at about 50° C., it then falls to less than 1 and finally becomes 0. In all these cases the organism was acclimatized to the desired temperature before actual measurements were made. With such precautions constant values of growth were obtained. The effect of temperature on the constant of growth has been determined in the case of many other cultures, but not over such a complete range of temperature.

J. E. Lane-Claypon (1909) measured rates of growth of *B. coli*, *B. typhosus* and *B. enteriditis* (Gaertner) in nutrient broth. The number of bacteria was estimated by ' plating ' and the influence of temperature was specially studied. The results are given in Table V.

TABLE V.

Temperature.	Generation Time.		
	B. coli.	*B. typhosus.*	*B. enteriditis.*
42 °C. ..	0·32 hours	—	0·37 hours
34·3 ,, ..	0·44 ,,	0·55 hours	0·51 ,,
31 ,, ..	0·53 ,,	0·75 ,,	0·56 ,,
26 ,, ..	—	—	0·71 ,,
25·5 ,, ..	0·86 ,,	—	—
25 ,, ..	—	1·30 ,,	—
20 ,, ..	1·30 ,,	1·67 ,,	1·00 ,,

In the case of yeast cells, the organism is large enough to be watched under the microscope and the effect of rising temperature observed. The experiment can be carried out by means of a ' warm ' slide. By timing the appearance of buds, generation times can be determined. As the temperature is raised, the G.T. becomes less, and at a critical temperature, about a degree higher than that at which steady growth occurs, a considerable disturbance in the development of the cells is noticed. Some die, others grow at the maximum rate, and some at a less rate. At a slightly higher temperature growth stops entirely. This is probably typical of the effect of high temperatures on the growth of micro-organisms.

Reaction of the Medium.

The reaction of the medium in which a culture of bacteria is developing is of considerable importance. Bacteria will not tolerate a very acid or a very alkaline medium. Most media are highly buffered solutions, and it seems that the pH of the medium, and not the titratable acidity, is of most importance. The effect of acidity on growth, therefore, resolves itself into finding relationships between growth and the pH of the medium. A definite acidity may kill the bacteria, thus Brünn (1913) found that *B. coli* dies within 24 hours in a medium of pH 4·7, but not of pH 5·0. C. G. L. Wolf and J. E. G. Harris (1917), in studying the growth of *B. sporogenes* in 2 per cent. glucose-peptone, found a definite acidity which

if exceeded inhibited growth, and if slightly less allowed growth. With a pH less than 4·94, growth was stopped, and with a greater pH growth took place. This seems to be a true acidity effect, for the reaction of the medium can be adjusted by hydrochloric acid, lactic, formic, acetic, or butyric acid, and practically the same critical pH is obtained.

Dernby (1921) gives the following acidities between which growth can occur and the most favourable acidity for growth of some of the common bacteria.

TABLE VI.

	Limiting pH.	Optimum pH.
C. diphtheriæ	6·0 to 8·3	7·3 to 7·6
B. coli communis ..	4·4 to 7·8	6·0 to 7·0
B. pyocyaneus	5·6 to 8·0	6·6 to 7·0
B. prodigiosus	5·0 to 8·0	6·0 to 7·0
Pneumococcus	7·0 to 8·3	7·8
Staphylococcus aureus ..	5·6 to 8·1	7·2 to 7·6

A correct reaction in the surroundings of a bacterium is essential in most cases to maximum growth and there seems little doubt but that alterations in pH as a result of bacterial fermentations are potent causes of limitation of growth. This fact is strikingly illustrated by alterations in bacterial flora, which may be induced in a mixed culture by varying the pH directly or indirectly by supplying food particularly desired by any one variety. The bacterial flora of the human gut may be entirely changed by this process (Cruickshank, 1928). In experiments on rats, Cruickshank finds that lactose and dextrin, when added to a diet of bread, meat and oats, quickly changes the intestinal flora to an aciduric type. Saccharose has a much less marked effect, and other carbohydrates produce no change. *B. acidophilus* becomes established as the predominant organism, probably owing to its acid-resistant property. Lactose and dextrin are slowly absorbed from the intestine and the attack on the available carbohydrate by the intestinal bacteria in the lower bowel produces an acidity which is inimical to all save the acid-tolerant group.

It may be remarked that many pathogenic bacteria have an optimum pH as estimated by rapidity and mass of growth on the alkaline side of the pH of blood, i.e. about pH 7·8 to 8·0. This pH would appear to be still more removed from that of the tissues, which are generally credited with a pH under 7·0. Where such conditions exist it is clear that the pH of the tissues must deter the organism from maximum activity.

An investigation of the effect of the reaction of the medium on growth is rendered more difficult by the fact that during growth the pH changes. The final reaction of the medium is influenced by the initial reaction, although the limits of acidity when growth ceases may be comparatively narrow. F. J. S. Wyeth (1919) allowed *B. coli* to grown in 2 per cent. glucose-peptone with the initial reactions varying between pH 4·30 to pH 9·82. The final reactions varied only between the narrow limits of pH 4·27 and 4·82. In 2 per cent. peptone the variation in the pH after

growth was much greater. Hagues (1929) has studied the influence of pH on the growth of various species of yeast. He finds that a torula shows a maximum rate of growth in wort at pH 5. *S. ellipsoideus* and *S. cerevisiæ* show two maximal rates of growth, one at pH 4 and one at pH 8·5. Two Pastorianus yeasts show in addition to these two maxima, a third one at pH 6·5. It is considered probable that the enzymes which take part in the growth of the cell show most activity at a definite pH, and it is at this acidity that growth is most rapid.

Surface Tension.

The surface layer of a medium in which bacteria are developing may differ appreciably in composition from that of the medium considered as a whole. It can, therefore, be expected that surface tension will play a part in the growth of bacteria, especially in the case of those developing on the surface of the liquid. This is found to be the case. Certain bacteria, such as *B. subtilis*, grow on the surface of the culture medium, and this pellicle growth is brought about by several factors. That air which is necessary for growth is specially abundant on the surface, is one factor, but not the only one. The bacteria themselves are denser than water, but do not sink owing to the surface tension of the liquid and to the fact that they are not easily wetted by the liquid. Pellicle-forming bacteria are specially rich in substances of a fatty nature, and remain on the surface for this reason. Substances like sodium ricinoleate in small quantities depress the surface tension and cause growth to take place in the liquid itself. Larson, Cantwell and Hartzell (1919) have studied the effect on bacterial growth of substances which lower surface tension, and specially noted the effect on surface growth.

Wolf (1923) discusses these results and gives a more detailed examination of the effect of small quantities of sodium ricinoleate on bacterial growth. He points out that the interface bacteria/water must also be brought into consideration. Interfacial tension is one of the factors which determine adsorption, and adsorption, no doubt, plays an important part in the growth of bacteria (Rideal, 1923).

Symbiosis and Antagonism.

Under natural conditions of bacterial growth more than one type of organism is usually present, and a study of the growth of mixed cultures is of considerable importance. In cases where the growth of one set of organisms is favourably affected or largely dependent on the presence of other organisms the term ' symbiosis ' is used. The amylo-process of fermentation is an example of this relationship. A mould which secretes diastase (*Rhizopus* Delemar) is grown on a starchy material and the sugar produced is fermented by a yeast (*S. anamensis*). In such cases yeast development is dependent on the growth of the mould, and could not occur to any great extent until the mould had converted the starch into a sugar assimilable and fermentable by yeast. Many other cases are

known where the products of metabolism of one set of organisms act as food for other organisms and a symbiotic relationship is established.

On the other hand, there may be antagonism between two races of bacteria developing in the same medium. Competition for food in some cases accounts for this interference in growth. Acidity produced by bacteria often suppresses the growth of others, thus *B. delbrücki* is used technically to produce lactic acid and inhibit the growth of undesirable bacteria. This is the case in the preparation of the mash in spirit manufacturing, and sometimes in lager beer brewing. Antagonism between organisms can usually be explained by competition for food or to the production of substances toxic to growth.

A familiar example of antagonism is the inhibition or overgrowth of *C. diphtheriæ* in cultures by *S. aureus*. Competition for food influences growth and in some cases differential growth in mixtures may be achieved by previous growth in the medium. For instance, Tulloch's exhaust medium favours *B. tetani* and inhibits *B. sporogenes* and the other actively proteolytic varieties which are responsible for the decomposition in the early stages of the preparation of the medium. The relationship between anaerobic bacteria growing simultaneously and influencing the growth of each other is of interest. The growth of aerobic bacteria in a mixture may be assumed to be the natural method for establishment of anaerobiosis.

M'Leod and Gordon (1923) and Burnet (1927) have grouped bacteria according to their power to produce hydrogen peroxide and according to the catalase contents of the cell. Anaerobic bacteria are sensitive to hydrogen peroxide and are helped by aerobic bacteria containing the enzyme catalase which decomposes hydrogen peroxide. Also bacteria which produce hydrogen peroxide retard the growth of other bacteria sensitive to this substance. In general, some of the antagonistic and some of the favourable influences which one growth of bacteria has on another can be traced to the production and decomposition of hydrogen peroxide in the manner indicated. Holman (1928), in discussing bacterial associations gives many examples taken from the literature, and points out what a very important part they play in many infections.

Suspended Animation and the Preservation of Cultures.

Growth of bacteria occurs only between certain limits of temperature. At high temperatures the organism ceases to grow and perishes quickly. At a temperature lower than that at which growth occurs, the bacteria perish only slowly and may live a long time. If a culture of bacteria is allowed to grow in a medium and then kept, the bacteria gradually die. The presence of products of fermentation such as acids hastens the death of the cells, and if such acids are neutralized by calcium carbonate the life is much prolonged. Yeast cultures kept in the fermented medium in which they have grown may survive 10 years or longer, a few cells still being alive after this time. Such suspended animation is of interest. Probably during this long period respiration of the yeast cells occurs, for

the life is shortened if air is excluded. Yeast cultures are sometimes preserved in sterile cane sugar solutions. In such cases life is probably preserved by a slow fermentation of the sugar. A short exposure of yeast cells to the temperature of liquid air does not kill the yeast, and at this very low temperature, true suspended animation no doubt takes place, for it is difficult to imagine any respiration or fermentation occurring under such conditions.

The problem of convenient methods of preserving cultures depends on a knowledge of the suspended animation of the organism. Reinoculation at fairly short intervals is the general method employed. Dried cells of organisms often exhibit prolonged life. In the case of yeast cells, if the yeast is dried after suspension in water and thorough aeration, the dried yeast contains a high proportion of living cells. Pressed yeast mixed with solid cane sugar liquifies, and the mass can be dried at a low temperature, the vitality of the main number of the cells being unimpaired.

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Cell-Division, Colony-Formation, and Spore-Formation.

By A. D. Gardner.

Cell-Division.

Methods of Observation.

The division and multiplication of bacteria can be satisfactorily studied by spreading the organisms on the surface of a flat block or film of nutrient agar, or similar material, and watching the growth with a high power of the microscope. If a cover-slip is placed over the bacteria an oil-immersion lens can be used (Hill, 1902 ; Graham-Smith, 1910 ; Hort, 1920 ; Oerskov, 1922 ; Gardner, 1925). Hanging-drop cultures are much less reliable, owing to passive or active movements of the organisms. When dried and stained preparations are employed as an additional aid, fixation of the wet film with osmic acid vapour before drying minimizes distortion and plasmolysis.

The Phenomena of Fission.

Planes of fission. The division of all elongated bacteria (bacilli, spirilla and vibrios) is transverse to the long axis. Longitudinal fission is unknown in bacteria proper, though it has been alleged to occur in spirochætes. Spherical organisms (cocci) sometimes elongate before division, and the fission then takes place transversely. A classification of the modes of fission and their results may be given as follows :

1. *Division in one direction.* Bacilli, spirilla, streptococci. If the new elements adhere at the point of fission, linear aggregates (false filaments) are produced.

2. *Division in two directions at right angles to one another, in one plane,* producing tetrads—*M. tetragenus* and sometimes *Gonococcus, Meningococcus* and allied cocci.

3. *Regular division in three directions at right angles to one another,* producing cuboid forms like roped bales—*Sarcina.*

4. *Haphazard division in any direction or plane,* producing groups of cocci of irregular form (including diplococci, bunches of cocci and short chains)—*Staphylococcus.*

Growth preceding fission. The fission of a bacterium takes place at the end of a phase of enlargement, and is conditioned by the size of the cell (or rather, of its surface) and by the quantity of soluble nourishment available. The rate of growth and fission is primarily dependent on the nature of the micro-organism. Thus, the first fission of a tubercle bacillus on a good medium at 37° C. takes about 48 hours at 37° C. (Miehe, 1909, confirmed by the writer), whereas that of a typhoid bacillus only needs 20 to 30 minutes. Secondarily, temperature and light (Ward, 1895) and, of course, nourishment, profoundly affect the rate of multiplication. In the early stages of a culture, when the medium is still fresh and rich, the cells attain larger dimensions than when the medium is approaching exhaustion (Clark and Ruehl, 1919 ; Henrici, 1925). In the later stages

of a culture (e.g. after about 12 hours' incubation of a rapidly growing bacterium) division keeps pace with growth and the great majority of the cells are relatively small and of fairly uniform size. (Wagner, 1898) put forward the theory that the true cellular unit of every species of bacillus has an approximately constant length, and that the longer individuals are really aggregates of such units. Several common facts of observation lend support to this view. For instance, the fact that, in spite of tradition and text-books, bacilli frequently divide into two very unequal parts is easy to explain in this way. Moreover, in stained preparations of bacilli it is often possible to make out a segmental structure in the longer cells with a regular periodic arrangement of granules and of the deep-staining areas of the cell-membrane.

The normal or typical cells of the ordinary (lower) bacteria are simple and unbranched (for the exceptions, see below under Irregular Division), whereas those of the *Actinomycetes*, which are usually classed as ' higher bacteria ', show frequent bifurcation.

Details of the process of fission. In most cases the first sign of division is a ring of constriction. This is followed by the growth of a transverse septum which, when complete, divides the organism into two halves (or fractions). In some of the larger bacteria septum-formation without constriction has been described (Dobell, 1911). In either case the septum then splits and the two halves can separate without any breach of the cell-membrane.

There is much variety of detail in different species. In the *corynebacteria* the fission is commonly incomplete, and the two new elements, adhering by an unruptured portion of the membrane, form an angular pair. The tubercle bacillus, growing on a solid medium under a cover-slip, very often bends to an acute angle, and the new growth pushes out from both limbs at the tip of the angle, without any visible septum having been formed (unpublished observations of the author).

Participation of supposed nuclear structures in division. In stained bacteria a number of observers have described small, deeply coloured, more or less central spherical or elongated bodies which they believe to be nuclei (Wagner, 1898; Nakanishi, 1901; Swellengrebel, 1906; Bredemann, 1909; Dobell, 1911; Douglas and Distaso, 1912; Park and Williams, 1917). But the evidence that such appearances are true nuclei in unconvincing. Constrictions in, or duplication of the so-called nucleus have been interpreted as amitotic nuclear division; but, again, this interpretation has not been accepted by the majority of bacteriologists. We do not, in fact, know enough about the internal structure of bacterial cell to give any account of the changes that occur in it during division.

Unequal Division.

The great majority of bacilli and spirilla produce, in certain circumstances, branched cells or Y-forms from which new bacilli can grow at all three branches or growing-points of the cell. This process was first

described by Hort (1920) in *B. typhosus*, and Gardner (1925), without knowledge of Hort's work, observed the phenomenon in a considerable number of species of bacilli and vibrios, and termed it ' three-point-multiplication ' (Plate I, Fig 2). It occurs chiefly in the fresh active growth from old cultures, and it seems to be connected with a temporary, or sometimes permanent alteration of the cell-membranes of the strain, due to deleterious surroundings. Individual colonies containing many Y-forms, in addition to numerous other abnormal shapes, may give birth to strains of permanently abnormal morphology, such as that shown at A in Fig. 4 of Plate I. True branching is thus a property of the whole class of the schizomycetes, and cannot be used as a distinctive mark of the *Actinomycetes*. Another form of irregular fission may be observed in bacilli of the typhoid-coli and other groups. A constriction appears close to the extremity of the rod, and a small spherical segment, like a single coccus, is set free. These ' gemmules ' are believed by some to be special reproductive bodies, but when put on fresh medium they do not, in the writer's experience, produce new bacilli. They seem rather to represent the shedding of an abortive and lifeless cell, or part of a cell (Plate I, Fig. 4B). Extremely unequal division is seen in many varieties of streptococci, which produce rods and spindle-shaped cells which were at one time erroneously called arthrospores. A species known as *Str. mutans* is particularly addicted to this atypical habit (Plate I, Fig. $4C_2$). Again, in old cultures of many bacilli or vibrios a considerable number of spherical or spheroidal elements may often be seen. The cholera vibrio, for instance, readily produces these ' coccoids ', which are often actively motile, and stain deeply with ordinary dyes. The writer has observed large numbers of them continuously in the living state, and has found that the majority undergo lysis when planted on fresh medium. A few, however, manage to hold on to life and slowly grow out into irregular bacteroids with a distinct tendency to branching. This peculiarity, which seems to occur in resistant individuals long exposed to bad conditions, can sometimes be perpetuated by selection (see Plate I, Fig. 3).

Post-fission Movements.

The changes that occur after fission determine the character of the resulting growth. Graham-Smith (1910) studied the growth of bacilli on agar blocks, and suggested a classification according to the character of the post-fission movements :

(1) *A loop-forming group*, of which *B. anthracis* is the representative. The membrane of this group is said to be very strong and adherent, so that the cells do not separate after division, but produce long looped filaments (Plate I, Fig. 1A).

(2) *A folding group*, into which *B. pestis* and some anthrax-like non-pathogenic bacilli are classed. Chains of bacilli are produced, with a soft, flexible capsular material joining the cells together, so that the chains bend into zig-zag shapes (Plate I, Fig. 1B).

Plate I (Description).

Fig. 1.—The post-fission movements of bacilli. A. Loop-forming group (*B. anthracis*). B. Folding group (*B. pestis*). C. Snapping group (*C. diphtheriæ*). D. Slipping group (*B. typhosus*). Successive drawings from life. After Graham-Smith.

Fig. 2.—The development of a colony from a single-branched cell (Y-form) of *B. dysenteriæ* (Shiga). The three limbs of the Y-form with the new bacilli springing from each are shaded differently. Successive drawings from life.

Fig. 3.—The development of an abnormal elongated cell and of a Y-form from a 'coccoid' cell in an old culture of *V. choleræ* on fresh agar. A similar coccoid is seen to have failed to develop. Successive drawings from life.

Fig. 4.—Irregular development and division. A. A four-hours-old colony of an abnormal pure-line *B. coli* which had a strong tendency to ballooning (as in drawing), autolysis and the production of branched cells. B. Formation of 'gemmules' by *V. choleræ*. When planted on fresh medium these invariably failed to grow. *C. Streptococcus* growing normally (C_1), and in bacteroid forms (C_2).

Fig. 5.—Rough (left) and smooth (right) colonies of the typhoid-coli group. Photographed by (A) reflected, (B) transmitted light.

Fig. 6.—The formation and germination of spores, semi-diagrammatic. 1. Successive stages of sporulation, as usually described. 2. A representation of spore-formation by coalescence of granules (after Hueppe). 3 to 6. Various modes of germination of spores, described in the text.

Plate I.

Fig. 1.

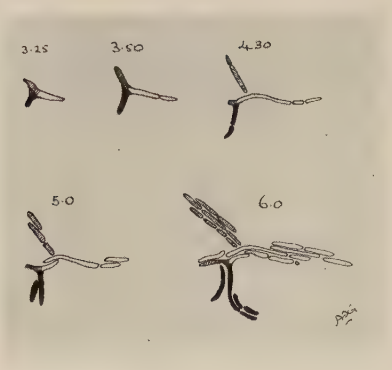

Fig. 2.

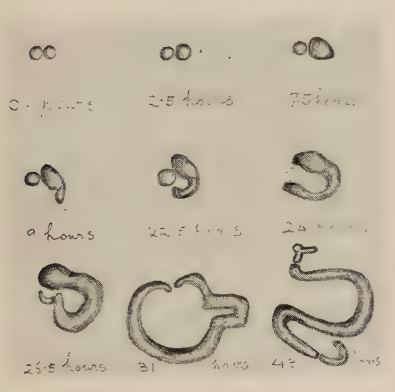

Fig. 3.

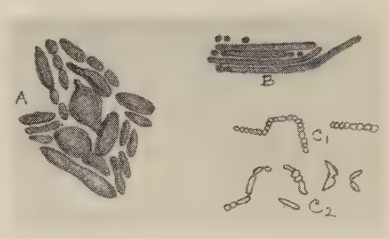

Fig. 4.

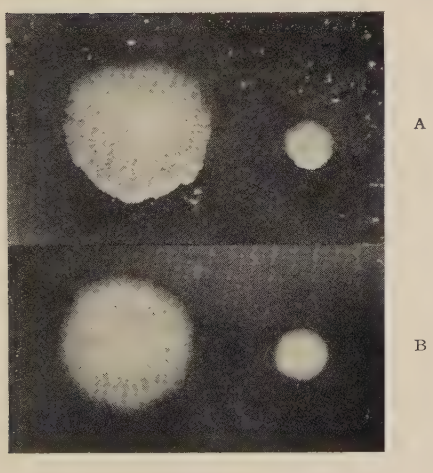

Fig. 5.

Fig. 6.

[*To face p.* 162.

(3) *The snapping group*, i.e. the diphtheria bacillus and the diphtheroids. Here the capsule partly ruptures, but the cells remain attached at one corner. The characteristic angular arrangement results (Plate I, Fig. 1c). *B. tuberculosis* divides in a similar way, and produces many angled pairs ; but its subsequent movements are ' slipping ', and give rise to compact, elongated wisps of growth.

(4) *The slipping group* includes the whole typhoid-coli-dysentery group, *B. pyocyaneus*, *B. proteus*, *B. subtilis*, vibrios, and many other organisms. Separation is complete, and the contiguous ends of the daughter bacilli slip past each other. The resulting growth consists of wisps of more or less parallel lines of cells (Plate I, Fig. 1d). It should be noted that this description is of the smooth type of growth only.

Colony-Formation.

Modes of Growth of Colonies.

Colonies may spring from a single bacterium or from a group of two or more. The appearance of the colony depends chiefly on (1) the chemical composition of the bacterium, e.g. dry waxy colonies of *B. tuberculosis* and other acid-fast organisms, pigmented colonies of numerous bacteria, and so forth. (2) The profusion of growth, which itself depends on the nutritive value of the culture-medium, and on the suitability of the gaseous environment. (3) The character of the post-fission movements which we have just described. For instance, Graham-Smith's loop-forming group produces ' Medusa-head ' colonies which a low magnification resolves into hair-like tresses. The smooth varieties of the slipping group produce uniform translucent colonies with a fairly regular circular margin (Plate I, Fig. 5). The rough modification, however, departs in varying degrees from the slipping type of movement. A bacillus in a completely rough condition, owing to the persistent adhesion of the ends of the cells after division, forms chains which fold in the manner described by Graham-Smith for his second group. The resulting colony is not of uniform density, and its edges spread irregularly (Nutt, 1927). The writer observed this phenomenon some years ago, but found it constant only in extreme cases. Colonies whose early growth showed the conventional slipping movements could nevertheless acquire a rough character when fully developed. Other factors, such as the altered constitution of the cell-membrane probably affect the appearance of the completed colony.

The production of rough and smooth colonies has been observed in many genera of bacteria in addition to those mentioned, e.g. in streptococci, pneumococci, gonococci and meningococci ; in *B. proteus*, *B. subtilis* and thermophilic bacilli (see Hadley, 1927).

Ring-formation on mature and ageing colonies is common. It may be due, as in the pneumococcus, to autolysis spreading behind the growing edge ; or, as in *B. paratyphosus B*, to the production of a heaped-up

slimy growth at the periphery (Schleimwall). Thinning, flattening and pitting of colonies are also caused by autolysis, and profound alterations may be effected by the action of Bacteriophage (see Vol. VII).

Circumscribed and spreading growth. On the ordinary agar or gelatin surfaces unlimited spreading of colonies is prevented by the gradual drying of the surface, and by the accumulation in the medium of metabolic products, which inhibit further growth. Where the layer of culture-medium is thick, the colonies tend to grow larger than where it is thin. Certain species of motile bacteria, however, such as *B. proteus*, spread so rapidly on the surface of a moist gel that instead of separate colonies a continuous film is formed. But here, too, modified non-motile forms of growth occur and give rise to circumscribed colonies (the ' O ' form of Weil and Felix, 1917). *B. tetani* often spreads on moist agar like *B. proteus* ; a peculiarity that has been found useful for its isolation.

Growth Stages in Colonies.

The growth of bacteria on solid media corresponds with their development in fluids. There is a preliminary lag-phase, in which the mature or resting cells are changing into embryonic or growing cells (Henrici, 1925) ; then a ' logarithmic phase ' of rapid growth ; and finally a stage of arrest, passing into a period of decline.

Owing to local shortage of nourishment, accumulation of metabolic products and, frequently, desiccation, the life of most of the cells of a colony is a short one. But some of the individuals adapt themselves to the deteriorating environment, and either continue to multiply slowly, or at least to survive ; with the result that the colony contains numerous living bacilli for days, weeks or months. Eventually the balance of life and death tilts more and more towards death, and after some months the colony has perished, or contains only a very few living elements. How it is that these few individuals are able to outlive their fellows so long, is at present a mystery, only to be explained by the pre-existence or the new birth of variants especially resistent to the adverse influences of the environment.

Secondary or daughter-colonies. On the colonies of numerous genera of bacteria, after a period of stationary senescence, little knobs or buds may be seen. Sometimes these daughter-colonies are able to ferment a carbohydrate in the culture-medium which the parent-growth had shown no power of attacking. *B. coli mutabile* and Sonne's type of dysentery bacillus provide two of the best studied instances of this phenomenon. The new lines cultivated from daughter-colonies usually preserve their apparently newly acquired faculties indefinitely, but may sometimes revert to the parent-type if subjected to powerful disturbing influences such as passage through animals. The ' new ' faculties of the daughter-colonies are, in all probability not really new, but merely remanifested after a period of suppression. In sporing bacilli daughter-colonies are only seen on colonies in which sporulation has occurred.

Spore-Formation.

General Description.

In a considerable number of species of bacteria under appropriate conditions the cells produce within themselves spherical or oval bodies whose structure and properties differ strikingly from those of the cells that produce them. These endospores are highly refractile and non-motile ; they resist the ordinary rapid staining processes, but are capable of being coloured by especially powerful methods (Vol. IX.). When exposed to harmful influences, such as heat, desiccation and a variety of chemical disinfectants, the spores manifest a power of resistance of an order vastly superior to that of the vegetative cells.

The development of a spore (Plate I, Fig. 6). In unstained preparations the first signs of sporulation are the cessation of growth and the appearance in the bacterial cell of a granule, which gradually enlarges and becomes more refractile than the remainder of the cell (Koch, 1877). At this stage the granule or spore-embryo takes up the ordinary bacterial dyes intensely. Soon, however, there appears around the granule a differentiated zone which develops into the spore-membrane. At the same time refractility increases to a maximum, and permeability to dyes diminishes greatly, so that the ordinary staining solutions can do no more than faintly tinge the outer surface of the membrane. Certain early workers (e.g. Ward, 1895) described the appearance of a number of refractile granules or droplets in the cell just before sporing, and a congregation and fusion of these bodies to form the spore-rudiment (Plate I, Fig. 6, 2).

The participation of chromatin granules or supposed nuclei in the formation of the spore has been alleged by various writers (Schaudinn, 1902, 1903 ; Dobell, 1911 ; Nakanishi, 1901 ; Gotschlich, 1927 ; Růžička, 1903 ; Ellis, 1922 ; Johnson, 1912 ; Kirchensteins, 1922 ; Petit, 1921) ; but we do not consider the evidence of the nuclear nature of these structures as satisfactory.

The spore gradually enlarges, and may achieve a diameter greater than that of the sporangium, or mother-cell ; in which case the resulting forms are known as clostridium (central spore) or plectridium (terminal spore). When the spore is ripe, the sporangium degenerates, and partially or entirely disintegrates, leaving the spore either naked or clothed with relics of cell-membrane.

Composition of spores. Apart from a high lipoid concentration, which is no doubt largely responsible for the impermeability of the spore and its optical properties, little is known of the composition of spores or of their membranes. It is probable that water is reduced to a minimum.

The Spore-Forming Bacteria.

Spherical bacteria (cocci) do not sporulate. Of the sporulating bacteria the great majority, perhaps all, are rectilinear rods (bacilli). Sporulating spirilla have been described, e.g. *Sp. sporiferum* (Migula), *Sp. endoparagogicum* (Sorokin) and *Sp. monospora* (Dobell), but their existence is not

universally accepted. None of the sporulating bacilli are pure parasites. Indeed, the very ability to form spores is proof of a potential existence outside the living body.

Variability of spore-production. Spore-bearing species may throw off variants which are temporarily or permanently incapable of producing spores (see especially *B. anthracis*). Indeed, Eisenberg (1912) believed all normal anthrax cultures to be mixtures of sporogenous and asporogenous races.

The Function of Spores.

The ability to form spores enables a bacillus to remain alive, even for many years (Székely, 1903), in the absence of food and water, and to resist wide variations of temperature, osmotic pressure and chemical action. The advantage of this to bacteria whose common habitat is soil needs no elaboration. An analogous process is found in the sporulating yeasts, and perhaps also in the encystment of protozoa. Most asporogenous bacteria can adapt themselves to a prolonged life of suspended animation in an environment incapable of supporting growth. In all probability profound inner changes of an unknown nature, and perhaps having something in common with the process of spore-formation, accompany this adaptation. No gross morphological changes occur, and resistance to heat and disinfectants is not increased ; but there is evidence that these surviving cells are considerably less easily killed by high osmotic pressures and by drying than are the cells of a young culture.

That multiplication is not the aim of sporulation is proved by the fact that a single bacterial cell seldom, if ever, produces more than one spore. It is true that certain giant bacteria have been observed to form two spores, and that the generic name *Dispora* has been given to them (Dobell, 1908). But it appears that, in these cases, the elongated double-sporing element really consists of two cells which have not yet separated.

The theory advanced by Schaudinn (1902, 1903) that sporulation is produced by a primitive sexual fusion (autogamy) of two sister-cells resulting from an incomplete division, was at first supported by Dobell (1908), but later (1909) opposed by him. Proof that anything like a sexual process occurs is still lacking (see Life-Cycles of Bacteria, p. 170).

Resistance of spores to heat, &c. All spores resist heat much better than the corresponding vegetative cells. Most species resist 80° C. in the wet state for at least some minutes, but are killed in 24 hours. A few spores in hay-infusions can withstand 70 to 80° C. for 3 or 4 days (Cohn, 1877). Boiling water at 100° C. is withstood for periods varying from a second to more than 24 hours, according to the species of bacillus, the nature of the material containing the spores, and the state of health of the spores. This latter varies considerably in the individual spores as well as in the mass (Flügge, 1894; Zettnow, 1912; Dannappel, 1899). According to Zettnow certain spores can actually survive dry heating to 300° C. for half an hour, but a similar period at 160° is usually enough to sterilize spore-contaminated

objects unless they are grossly dirty. This remarkable power of heat-resistance can only be explained by assuming that spores contain the least trace of water compatible with life. Steam at temperatures over 100° C., i.e. under pressure, is rapidly fatal to naked spores, but if they are contained in anhydrous fluids, such as oil and glycerin, autoclaving fails to kill them, presumably because they are protected from the penetration of the steam. In these conditions a temperature of 170° C. for half an hour is needed to kill spores of *B. subtilis* (Dreyer and Walker, 1912 ; Bullock, 1913). The resistance of spores to high osmotic pressures, and to relatively high concentrations of chemical substances that are lethal to the vegetative cells, is attributable to the impermeability of the dense spore-membrane. Variation in heat-resistance occurs spontaneously, or may be induced by change of culture-medium, or of incubation-temperature (Williams, 1929).

Length of life. Spores are by no means immortal. Swann (1924) found that 5 per cent. of young anthrax spores (one week old) failed to germinate. Dried-up cultures 12 months old contained less than 50 per cent. of living spores.

Conditions Affecting Sporulation.

Bacteria sporulate most freely when, at the end of a period of intense multiplication in a rich medium, the conditions have ceased to be favourable to vegetative growth (Gaertner, 1903 ; Holzmüller, 1909). Gradual drying, such as occurs at the upper, thin end of an agar slope culture, or in fluids exuded from the carcass of an animal lying in the open, is an important stimulus. Deprivation of food, by suspending the bacteria in distilled water or weak mineral solutions (Schreiber, 1896), also favours sporulation. Holzmüller (1909) states that oxygen acts on obligate anaerobes as a direct stimulus to spore-formation ; and in the anaerobic *B. aerogenes capsulatus*, Fitzgerald (1911) believed that the presence of at least traces of oxygen was necessary to initiate the process. But it seems impossible to admit the necessity of oxygen, since it has been shown that in a well-grown and sporing culture of an anaerobic bacillus there may be less than one molecule of oxygen in 10^{13} litres of culture. In aerobic bacilli, anaerobic conditions inhibit sporulation as well as growth.

If an organism has been growing slowly in somewhat unfavourable circumstances, such as a temperature above the optimum, or a pH above or below it, spore-formation is inhibited to various degrees.

Germination of Spores (Plate I, Fig. 6).

When a spore is placed in a suitable nutrient medium it begins to increase in size (Ward, 1895 ; Holzmüller, 1909 ; Bredemann, 1909), ruptures or absorbs its capsule, and elongates into a new vegetative cell.

There is a considerable degree of constancy in the mode of germination of the spores of a species—a fact that can be utilized in the differentiation

of bacteria. But there are often irregularities, not all individuals conforming exactly to the rule. Marshall Ward (1895) describes the germination of a spore of *B. ramosus* (a water saprophyte) in the following words : ' Under the conditions referred to, the spore without materially changing its ovoid shape begins to swell somewhat rapidly, and in from one to two hours has increased its dimensions from $1 \cdot 5 \times 2 \cdot 0 \mu$ to $2 \cdot 0 \times 2 \cdot 5 \mu$ or more. As it does this the brilliant oil-drop-like contents become duller and more hyaline—like ground glass—and the sharply marked, almost black membrane gradually loses some of the firmness of its contour, until it appears as a thinner limiting membrane. At the same time it becomes surrounded by an almost imperceptible, pale, halo-like investment which appears to be derived from the deliquescence of its most external layers. In the course of the next one to two hours or so, the spore appears to be elongating. Close observation shows this to be due to a thinning out of the membrane at one of the ends, and soon afterwards the thinned-out wall gives way, and the pale hyaline, apparently homogeneous protoplasm . . . pushes its way out, and grows as a blunt rod in the direction of the long axis of the spore '. The germination of *B. anthracis* is generally polar, and takes place as follows. The spore first loses its strong refractivity, and begins to elongate. At this stage it may take up aniline dyes intensely. Then it splits at one end, and the new bacillus grows out from the still adherent spore-case. *B. subtilis*, on the other hand, germinates equatorially. The spot at which rupture most commonly occurs is said to be discernible beforehand as a small elevation of the membrane (Nakanishi, 1901). In the group of *B. mycoides* polar germination of the ovoid spores is the rule (Holzmüller, 1909), but exceptions are numerous. Bipolar rupture is often seen, the new bacillus growing out at both ends; or a lateral bursting of the membrane takes place with a looping outgrowth of the centre of the new bacillus, whose ends are impacted in the spore-case. In certain species of this group, e.g. *B. dendroides*, the cylindrical spores appear to elongate into bacilli without visible rupture.

Dormancy. When spores are planted on a nutrient medium suitable for vegetative growth, the great majority germinate rapidly, but a small proportion may lie dormant for varying periods before germinating. For example, Burke, Sprague and Barnes (1925) observed dormancy ranging from a few days to three or four months in spore-platings of *B. subtilis* and *B. megatherium*.

Conditions affecting germination. Holzmüller (1909) found that spores of the *B. mycoides* group would only germinate within a narrow range of osmotic pressures (salt content) corresponding to the optimum for vegetative growth. The same group shows a fairly wide range of temperatures at which germination occurs, i.e. between 8 and 53° C., but at the extremes of the range it is imperfect, and it only takes place with full freedom at the optimum range of 28 to 35° C. Other species doubtless have similar though not identical optimums. Exposure of spores to direct

sunshine usually prevents their germination (Ward, 1895; Schreiber, 1896). Holzmüller found some species of the *B. mycoides* group to be similarly affected, while others proved able to germinate in direct sunlight under running water. Ward (1895) showed that the deleterious action of light is exercised by the shorter waves.

Germination-period. Working with *B. anthracis*, Swann (1924) found that the germination-period of young anthrax spores at 37° C. is short (about 85 minutes at 37° C.), constant, and independent of the age of the spore, up to 47 days, provided the culture be moist. Old spores in dried cultures show a minimum of two hours and a maximum of seven hours. The old type is usually distinguishable from the young by its smaller size, and less ' juicy ' appearance. Owing to the impossibility of deciding the moments at which germination proper begins or ends, the germination-period was defined as the time between the beginning of incubation and the first division of the germinated cell.

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The Life-Cycles of Bacteria.

By H. G. Thornton.

Introduction.

From the middle of the nineteenth century, investigators have held opposing views as to the stability of cell form amongst bacteria and as to their methods of reproduction. The opinion most commonly held dates from the writings of Cohn, Koch and other early investigators. According to this view each bacterial species has a normal cell shape, from which it shows little deviation on standard media and under active conditions of growth. Multiplication is said to occur only asexually by fission into two equal-sized daughter-cells. This simple conception, upon which existing systems of classification are based, was attacked as early as 1877 by Nageli and his associates, but their views were too extreme for general acceptance. Within the last twenty-five years, however, a number of investigators claim to have shown that bacteria undergo sudden changes to entirely different cell shapes and that they multiply by other methods in addition to binary fission. The writings of Löhnis (1921), Hort (1917), Enderlein (1925), and Mellon (1926) have given especial prominence to this view, and an increasing number of investigators has given it entire or partial support.

Unfortunately, the methods used by many of its supporters have exposed their opinions to criticism, mainly directed along two lines. It is held first, that investigators have not always demonstrated the purity of their cultures, so that the appearance in them of new types of cell may be the result of contamination, and, secondly, that some of the appearances described may be due to unsuitable conditions of growth, or even to death and disintegration of the cells. To demonstrate the existence of sudden changes in cell shape, these should either be shown to take place in cultures of undoubted purity derived from single cells, or, better, should be followed by observing individual cells under microscope. Methods of reproduction should be studied by following the actual formation of new cells. There are, however, certain instances in which the observation of a complete series of intermediate stages provides strong evidence of a life-cycle process, though the piecing together of a cycle from such observations is usually dangerous.

The changes in morphology that have been described fall into three distinct categories. There is firstly the appearance of an entirely different cell type which can often be isolated and kept through a large number of subcultures, after which it may revert to the original cell type or may change into a further type. Secondly, there are gradual changes in the shape of the vegetative cells which occur during the growth of a single culture and are related to its physiological age. And thirdly, there are phenomena connected with various methods of reproduction.

Sudden Appearance of New Cell Types.

The most extensive series of studies in bacterial life-cycles are those of Löhnis and Smith (1923), dealing mainly with strains of *Azotobacter*. This organism may therefore be taken as an example.

Azotobacter occurs typically in the form of oval cells (2 to 3 × 3 to 5μ) enclosed in thick gelatinous capsules. Löhnis and Smith claim that this cell type gives rise to the following forms, which can be isolated in pure culture :

(1) Micrococcus forms, 0·3 to 1·0μ diameter.
(2) Oval, wedge-shaped or rod-like cells, 0·3 to 0·75μ long.
(3) Rod-shaped cells (0·3 to 0·5 × 0·75 to 1·0μ), sometimes motile, which do not form endospores.
(4) Similar small rods which produce endospores.
(5) Large spore-forming rods.
(6) Branching fungoid forms.

Sometimes it was easy to isolate the new form in pure culture, but in other instances the small numbers of the new type present, or its tendency to revert to the original type made isolation difficult. The new cell types differ not only in morphology but in the appearance of their growth, in pigment formation and in their physiology. The authors did not use cultures isolated from single cells, but claim, on the following grounds, that the new types were not contaminations. (1) The purity of their cultures was frequently tested by plating in flat bottles, used to minimize the chances of air infection. (2) In many cases stages in the production of a new cell type could be found. (3) The same six new cell types in addition to the typical large oval cells appeared several times in each of the 30 strains studied. (4) All the six new cell types eventually gave rise to the normal large oval cell type. The probability against this happening by chance contamination is very large. The number of times each cell type gave rise to the other forms is shown in Table I, in which some of the morphological types are further subdivided according to pigment formation. Löhnis and Smith's results with *Azotobacter* have been partially confirmed by Jones (1920) and by E. Petersen. The latter, in a series of observations not yet published, obtained the micrococcus, short rod, and branching fungoid forms from single cell cultures. He isolated and grew these forms in pure cultures, but has not yet succeeded in

making them revert to the oval cell form. He was able in some cases to follow the development of the new cell types by watching individual cells under the microscope. There is thus strong evidence for the existence of several distinct cell forms in *Azotobacter*. There remains the question

TABLE I.

Types of growth and transformations observed in strains of *Azotobacter* (Löhnis and Smith, 1923).

	Number of Transformation to Different Types of Growth.												
	Large Spore-free Cells.	Coccoids.			Dwarfed Growth.	Spore-free Small Rods.		Fungoid Growth.			Sporulating Rods.		Total.
		White.	Yellow.	Red.		White.	Yellow.	White.	Yellow.	Orange.	Small.	Large.	
Large spore-free Cells.	—	1	2	2	4	4	1	9	0	0	5	7	35
Coccoids :													
White ..	3	—	1	0	3	3	0	3	0	0	3	1	17
Yellow ..	1	2	—	0	2	3	0	0	3	1	0	0	12
Red	1	0	0	—	0	0	0	0	0	0	0	0	1
Dwarfed Growth	2	1	1	0	—	2	3	1	6	1	1	0	18
Spore-free Small Rods :													
White ..	4	5	4	0	4	—	0	1	1	1	2	1	23
Yellow ..	0	0	0	0	1	0	0	2	0	0	0	0	3
Fungoid Growth :													
White ..	3	5	3	0	4	5	1	—	1	1	3	1	27
Yellow ..	0	0	0	0	0	0	1	0	—	0	0	0	1
Orange ..	0	1	0	0	1	2	0	0	1	—	0	1	6
Sporulating Rods :													
Small ..	2	3	1	1	2	2	0	4	0	1	—	4	20
Large ..	5	1	4	0	4	3	0	3	0	1	4	—	25
Total ..	21	19	16	3	25	24	6	21	14	6	18	15	188

as to whether this 'pleomorphism' is of general occurrence amongst bacteria or whether *Azotobacter* is unusual in this respect. Löhnis and Smith and other investigators, such as Cunningham and Jenkins (1927), Barber (1907), Stamm (1914) and Fürst (1914), have described similar changes in other bacteria, in some cases using cultures obtained from

single cells, while Löhnis, in an extensive search of literature claims that ' pleomorphism ' has been described and illustrated by many investigators who did not always interpret their observations correctly (1921). It is likely that bacterial species differ in the frequency with which they give rise to new cell forms and this may account for such forms having been overlooked in many cases.

The sudden appearance of a new cell type which will persist in culture for many generations is comparable with the sudden change in colony form or biochemical activity which has been described in many species

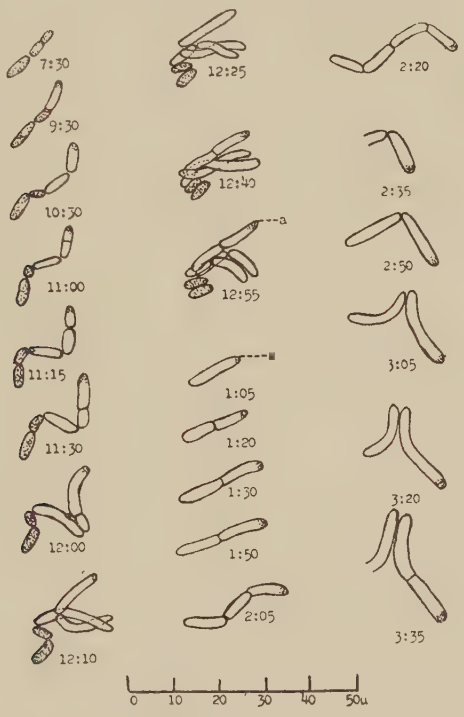

FIG. 1. Morphological changes in a growing colony of *B. megatherium* (Henrici, 1928).

and is sometimes termed ' dissociation '. The phenomenon has been given the name ' mutation ', though this suggests a knowledge of the genetics of bacteria which we do not possess. But the appearance of a new cell form which can be isolated and will remain constant in subcultures for a large number of generations bears a closer analogy to the changes studied by geneticists than to the life-cycles in other organisms such as protozoa, which are concerned mainly with methods of reproduction and conjugation, and which are usually completed in a comparatively small number of generations. It seems unfortunate, therefore, that the term ' life-cycle ' has been made to include this appearance of a new type of vegetative cell.

Changes in the Vegetative Cells Related to the Physiological Age of the Culture.

It has long been recognized that the mean size and shape of vegetative cells in a pure culture differ according to its age. A detailed quantitative study of these changes has been made by Henrici (1928), who divided the life of a culture into three stages, (*a*) the embryonic, (*b*) the adult, and (*c*) the senescent (Fig. 1). The embryonic stage is characteristic of the period of logarithmic increase, when the bacteria are multiplying most rapidly. The cells are usually larger than in the adult phase and stain more evenly. As the multiplication rate slows down, the bacterial cells gradually assume the adult phase, in which the cells assume the appearance usually regarded as characteristic of the species. In the senescent stage, the so-called involution forms begin to appear. The series of changes in the life of a culture of bacteria is comparable with the phenomena of youth, maturity and age in metazoan cells, and is quite different in nature from the sudden appearance of a new type of cell which may persist through a large number of subcultures, such as was discussed in the last paragraph.

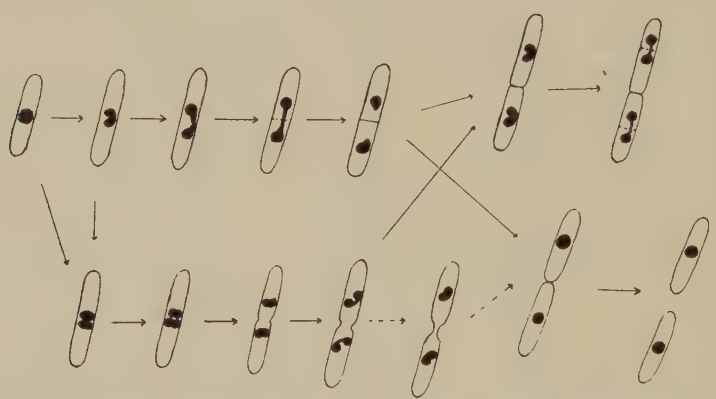

Fig. 2. Apparent nuclear changes in *B. malvacearum* (Stoughton, 1929). (Reproduced with the permission of The Royal Society.)

Changes Associated with Reproduction.

The question as to the presence and nature of a formed nucleus in bacterial cells is fundamental to any discussion of bacterial reproduction. A definite body behaving like a nucleus has been described in a number of species by such investigators as Dobell (1911), Mencl (1907), Guilliermond (1907), Enderlein (1925) and Stoughton (1929) (see Fig. 2). Mitotic division has been claimed to exist by Mencl (1907), Guilliermond (1907) and others, but the minute size of the structures brings them so near the limits of optical resolution that such observations are difficult to interpret. The resting nucleus proper consists, according to Enderlein, of a minute granule which he terms a ' mychit ', several of which may occur in one cell. This body is as a rule surrounded by stainable food material, as also occurs in many protozoa.

In addition to the commonly observed multiplication by binary fission a number of investigators claim that other forms of reproduction occur. The reader is referred to Löhnis's monograph (1921) for reference to such observations. The work of Löhnis and Smith (1916, 1923) principally with *Azotobacter*, and of Hort (1917–20), who studied several pathogenic bacteria, using a single cell technique, have drawn especial attention to this subject. The work of some authors is open to the criticism that the viability of supposed reproductive granules and buds has not been established, but in certain cases critical work seems to have proved the existence of reproduction by these means.

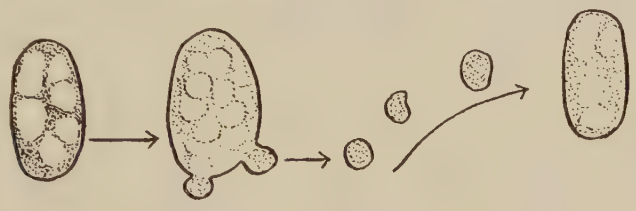

FIG. 3. Reproduction by budding in *Azotobacter*.

Budding.

The formation of buds has been observed by numerous workers. They have been described in *Azotobacter* (Fig. 3) by Löhnis and Smith (1916, 1923), Jones (1920) and by E. Petersen. Hort (1917) followed the development of buds in the colon-typhoid group, while recently Stoughton (1929) has described the formation of buds in *B. malvacearum*, but he has not yet seen these develop into the typical cell form.

FIG. 4. Reproduction by gonidia in *Azotobacter*.

Formation of Gonidia.

Granules claimed to be reproductive and termed 'Gonidia' by Löhnis have been seen to arise in bacterial cells of normal shape, and also in enlarged or encysted cells. They are released either by rupture of the mother-cell or by extrusion. The formation and release of these bodies in *Azotobacter* as described by Löhnis and Smith (1916) is shown diagrammatically in Fig. 4. E. Petersen followed the formation of both buds and 'gonidia' in this organism and saw their subsequent growth by watching individual cells. The growth of similar granules in the colon-typhoid group was similarly followed by Hort (1917). The finding of intermediate stages seems to demonstrate the reality of this method of

reproduction in some other instances. Thus, in *B. radicicola* the rod-shaped cells at first stain evenly, but later the stainable material becomes segregated into bands. These develop into spherical granules of which a single cell may contain from 1 to 6. Rupture of the mother-cell releases the granules, which at first are usually non-motile, but later swell in size and become actively motile (Thornton and Gangulee, 1926). The derivation of these motile cocci from the banded rods is shown by the occasional development of flagella upon the cocci before their escape from the mother-cell. All stages in the elongation of the cocci to form the evenly staining rods can also be found (Fig. 5). ' Gonidia ' are often of very small size so that indirect evidence of their existence has sometimes been obtained by filtration experiments.

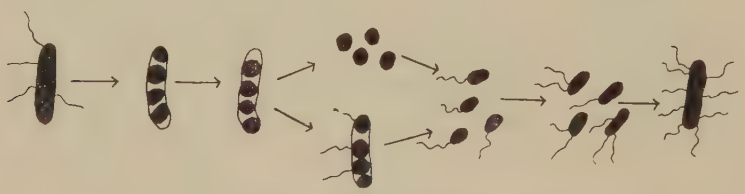

FIG. 5. Reproduction by gonidia in *B. radicicola*.

Endospores, Arthrospores and Microcysts.

The inclusion under the term ' spores ' of all heat-resisting bodies produced by bacteria regardless of their mode of formation, seems confusing. The ' gonidia ' have at least an equal right to this term, since they can be compared with the ' ascospores ' of fungi. The formation of resistant endospores is too well known to need description. They may perhaps be regarded as ' gonidia ' that have become surrounded by a resistant envelope. Löhnis suggests that the term ' arthrospore ' should from its etymology be confined to cases where fragments derived by the fission of the original cell become surrounded by a resistant coat. He suggests the term ' microcyst ' for whole cells which have been made more resistant by thickening of their cell-walls. Such cysts are analogous to encysted forms of protozoa and may give rise to normal cells by germination, or the contents may give rise to ' gonidia ' as has been described in *Azotobacter*.

Conjugation.

The existence of conjugation amongst bacteria is a debated question. Löhnis claims that a direct union between individual cells is of common occurrence and that it is characteristic of cultures 2 to 4 days old. As a rule two cells unite, but since more than two may do so, he considers the terms ' copulation ' and ' conjugation ' to be misleading and suggests the name ' conjunction '. In some bacteria, tube-like processes are seen connecting two cells. A good example of this has been described by Potthoff (1924) in *Chromatium* where the large size of the cells makes

phenomena easy to follow (Fig. 6). Such bridges are not always formed, and in most cases it is difficult to be certain whether the pairs of cells are the result of a union of originally separate cells or whether they are late stages of cell division. Cells in conjunction can often, according to Löhnis, be distinguished from division stages by their characteristic habit of lying at an angle to one another. Until complete fusion of the cells or the passage of material from one to the other can be demonstrated, it is difficult to be certain that their positions in contact are not due to chance distribution of the cells in the suspension or film. Enderlein claims to have observed the copulation of gametes derived from gonidia. Much critical observation is still needed to establish the existence of a sex process amongst bacteria, although analogy with other unicellular organisms leads one to expect it.

Fig. 6. Cells of *Chromatium okenii* in conjunction (Potthoff, 1924).

Symplasm.

It has been claimed that bacteria, in addition to their existence as individual cells, may fuse together to form a syncytium. Löhnis, the chief advocate of this view, calls this condition ' Symplasm ' and describes its formation as follows : ' The formation of symplasm always takes place in two phases : first, the bacteria agglomerate to smaller or larger clumps ; second, the cells dissolve more or less completely to a crumbly or slimy mass, assuming the shape of flakes, irregular clumps, or regular spheroid bodies, and being sometimes endowed with amœboid movement. Apparently all species of bacteria and all kinds of vegetative cells as well as reproductive organs, are able to enter the symplastic stage. It occurs in both young and old cultures, dependent on internal as well as on external conditions '.

In *Azotobacter*, Löhnis and Smith did not observe amœboid movements, but did see streaming movements within the symplasm. Sometimes the symplasm is naked and sometimes it is enclosed in a cyst-wall. Its staining properties also vary. It is claimed that new bacterial cells or reproductive bodies such as gonidia are formed by segregation of the symplastic material.

More exact information as to the mode of formation of new cells in the symplasm is needed before the claim that it is a syncytium can be established. A critic naturally questions whether the symplasm may not be a mass of slimy secretion or consist of dead cells that have undergone

decomposition. The possibilities seem difficult to exclude even if the observations are correct, since the development of new cells within the mass might be due to the presence within it of previously formed gonidia. The streaming and amœboid movements are physical phenomena not necessarily associated with living protoplasm.

Conclusion.

The sudden appearance of a new cell type capable of propagation in a pure state and reproduction by buds and gonidia seems to have been demonstrated in certain bacteria. The evidence for the existence of a sexual or ' conjunction ' process and of a syncytial stage is less convincing, since many of the observations seem capable of other interpretations.

It is difficult to predict the extent to which present-day bacteriology will be affected by the study of life-cycles until an extended series of critical observations has shown whether the changes are of general occurrence or whether they are confined to certain aberrant groups. If they are a general property of bacteria, existing systems of classification will be undermined. It may be possible to bring the classification of bacteria more into line with that of fungi and protozoa, by basing it upon the details of the reproductive processes and upon the morphology of the different cell types produced. If pathogenic and other bacteria of economic importance are liable to sudden changes not only in morphology but in physiology, this must greatly affect medical and other applied aspects of bacteriology.

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CHAPTER V. THE THEORY OF DISINFECTION.

By H. Chick (Lister Institute).
With a Section by C. H. Browning (University of Glasgow).

Introduction.

The modern concept of disinfection dates from the last decades of the nineteenth century, with the discovery of Pasteur that putrefaction is caused by the activity of micro-organisms and can be prevented if these are eliminated by chemical or physical means. Lister perceived the significance of Pasteur's discovery for surgery and showed that the 'putrefaction' and suppuration of wounds are the result of infection with microbes, and can be prevented by treating everything coming in contact with the wound with disinfectants. Following the work of these two pioneers, came numerous researches upon the subject; they were devoted for the most part to estimation of the relative value of the then existing disinfectants, and to the discovery of new ones. Among the authors of these investigations may be mentioned the names of Esmarch, Henle, Fraenkel, Geppert, Behring and Robert Koch. The work of Koch (1881) is specially noteworthy in pointing out the ineffectiveness of most of the disinfectants up to that time relied upon for the destruction of spores, and in demonstrating the value of mercuric chloride for this purpose. But he also was the first to demonstrate the effect of rise in temperature in hastening the action of disinfectants, and the great difference existing between the concentrations required to inhibit the growth of bacteria and to kill them.

The Process of Disinfection.

Observed Facts relating to Time-Survivor Relationships.

Krönig and Paul in 1897 made the first attempt to study the process of disinfection and discovered that the action does not take place suddenly, but is a gradual operation, in which the number of bacteria killed in unit time is greater at the beginning and less and less as the disinfection proceeds. In the twenty years which have elapsed since their work, others (Madsen and Nyman, 1907; Chick, 1908, 1910; Paul and his co-workers, 1909, 1910; Smith, 1921, 1923) have attacked the same problem, and although the technique has been different, the method has, in general, been the same, viz., the exposure of a measured number of living bacteria to some disinfectant and enumeration of the survivors by means of plate cultures from time to time until disinfection is complete.

If a general survey is made of the results obtained by these different workers, and curves are drawn in which the number of survivors in unit volume is plotted against time, the points are found to lie upon smoothed curves (curve a in Fig. 1) indicating that some definite numerical relation exists between the two variables. The shape of these curves is not, however, uniform [see Fig. 2 (a)] and the conclusions drawn by different workers, after mathematical analysis of their own results and those of others, has led to differences of opinion upon the nature of the disinfection process and upon the circumstances determining its course. These will be discussed below after a short summary of the facts has been given.

The results of Krönig and Paul's experiments, in which anthrax spores dried in a uniform film upon garnets were exposed to the action of mercuric chloride, were analysed ten years later by Madsen and Nyman (1907) and by Chick (1908), who found that the logarithm of the number of bacteria surviving in unit volume was proportional to the time which

TABLE I.

Disinfection of Anthrax Spores with 5 per cent. Phenol at 33·3° C. (Chick, 1908).

Sample No.	Amount of Sample.	Time elapsing, Hours.	Nos. of Bacteria counted, Mean of 6 Plates.	Mean No. of Bacteria present in 1 drop Disinfecting Mixture.	Log n*.	K, assuming Reaction to be in accordance with the Equation $-\frac{dn}{dt} = Kn$ $\left(K = \frac{1}{t_2 - t_1} \log \frac{n_1}{n_2}\right)$
1	1 drop	0	439	439, taken as initial value of n $(= n_1)$ in calculating values of K.	2·64	—
2	1	0·5	275·5	275·5	2·44	0·40
3	1	1·25	137·5	137·5	2·14	0·40
4	1	2	46	46	1·66	0·49
5	2 drops	3	31·6	15·8	1·20	0·48
6	2	4·1	10·9	5·45	0·73	0·46
7	4	5	13·9	3·6	0·56	0·41
8	6	7	3	0·5	—0·31	0·42
						Mean value of K, 0·44.

* In this and subsequent tables, logarithms reckoned to the base $_{10}$ are used in place of natural logarithms.

had elapsed, so that, when one was plotted against the other, the experimental points lay on a straight line* [see Fig. 2 (b), Curve A[1]].

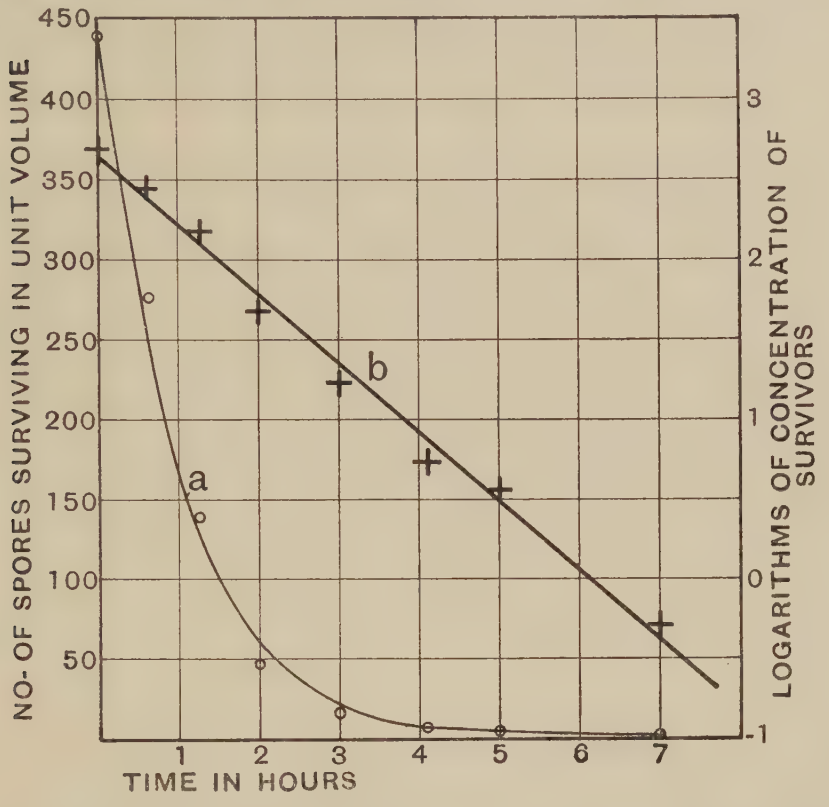

FIG. 1.—Disinfection of anthrax spores with 5 per cent. phenol at 33·3° C. (Chick, 1908). Curve a. Survivor-time curve; the number of surviving spores in unit volume plotted against time. The curve is drawn through a series of calculated points, the circles show results of experimental determinations. Curve b. Logarithms of concentration of survivors plotted against time.

* Disinfection proceeding in this manner appears to obey the Mass Law and to simulate what is known by chemists as a ' unimolecular reaction ' or a ' reaction of the first order '. The equation $\frac{-dC}{dt} = KC$

$$\text{or, } \frac{1}{t_2 \cdot t_1} \times \log. \frac{C.1}{C.2} = K \text{ (a constant)}$$

is applicable to the process of disinfection if, in place of the terms (C_1C_2) expressing concentration of reacting substance, numbers of surviving bacteria are inserted thus: $\frac{1}{t_2 - t_1} \times \log. \frac{N.1}{N.2} = K$, where N.1 and N.2 are the number of surviving bacteria (in unit volume) after times t_1 and t_2 respectively.

While anthrax spores are being killed by mercuric chloride, the number of spores perishing in any small interval of time (disinfection rate) is a constant proportion of the total number surviving at that moment. In other words, successive intervals of time form the terms of an arithmetical progression while the numbers of bacteria surviving at these times are ranged in a geometrical progression.

Disinfection of anthrax spores by phenol was found to proceed in a similar manner by Chick (1908), using suspensions of spores. In Table I are given the result of one such experiment; the values of K, the velocity constant, in the last column, exhibit fair approximation to a constant value. Fig. 1, Curve b, shows the experimental points ranged upon a straight line when time is plotted against logarithms of numbers of survivors.

Provided that precautions are taken to secure for the test as uniform a bacterial population as possible, this logarithmic relationship between survivors and time has been found to hold for disinfection with hot water of anthrax and *B. subtilis* spores (Madsen and Nyman, 1907; Eijkman, 1912) and of the following vegetative organisms, *B. coli*, *B. typhosus*, *B. pestis* (Chick, 1910); for death of *B. typhosus* and *B. coli* by weak acid (Cohen, 1922); for death of *Staphylococcus* by drying or by action of hydrochloric acid (Paul and co-workers, 1909, 1910); for destruction by hot water of the alga *Chlamydomonas* (Harvey, 1909). The results of Clark and Gage (1903) upon the disinfectant action of sunlight have proved on analysis to show the same relationship, and, recently, Burbury (1928) has found that the destruction by heat of the filterable virus of Foot-and-Mouth Disease in blood at 50° C., also proceeds in accordance with the Mass Law.

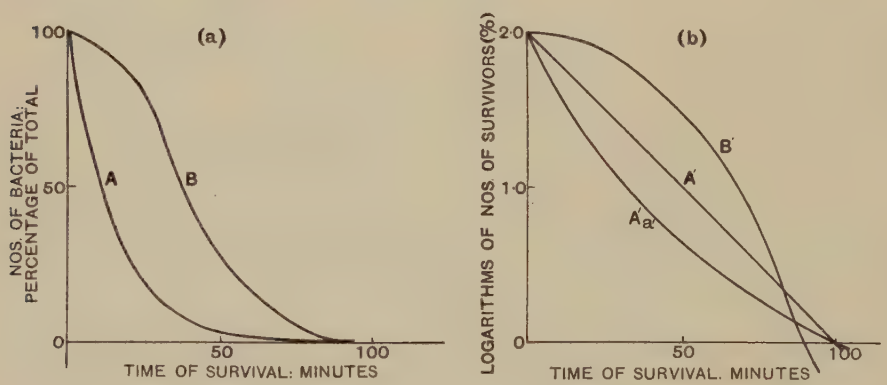

FIG. 2.—(a), (b). Time-survivor curves expressing the disinfection process.

In (a) the number of survivors in unit volume (expressed as per cent. of total); in (b) the logarithms of these numbers are plotted against time.

Curves A, A^1 refer to processes in accord with the Mass Law.

Curve A^1a^1 refers to processes in which bacteria are killed more quickly at first than if the Mass Law were obeyed.

Curves B, B^1 sigmoid curves.

There are many other instances, however, where the disinfection process, as studied by enumeration of the survivors at succeeding intervals of time, does not fall in with the mathematical scheme outlined above and expressed by curves A and A^1 in Fig. 2. Sometimes the death-rate at the beginning is *quicker* than would be the case if the numbers killed in unit time were proportional to the existing living population, and the

logarithms of survivors plotted against time lie not upon a straight line but on a curve concave to the axis [Curve A′a′, Fig. 2 (b)], and the velocity constant K diminishes continually as disinfection proceeds. Examples of such disinfection are that of *B. paratyphosus* by heat or phenol (Chick, 1908, 1910), and of a ' large sporing bacillus ' investigated by Eijkman (1912).

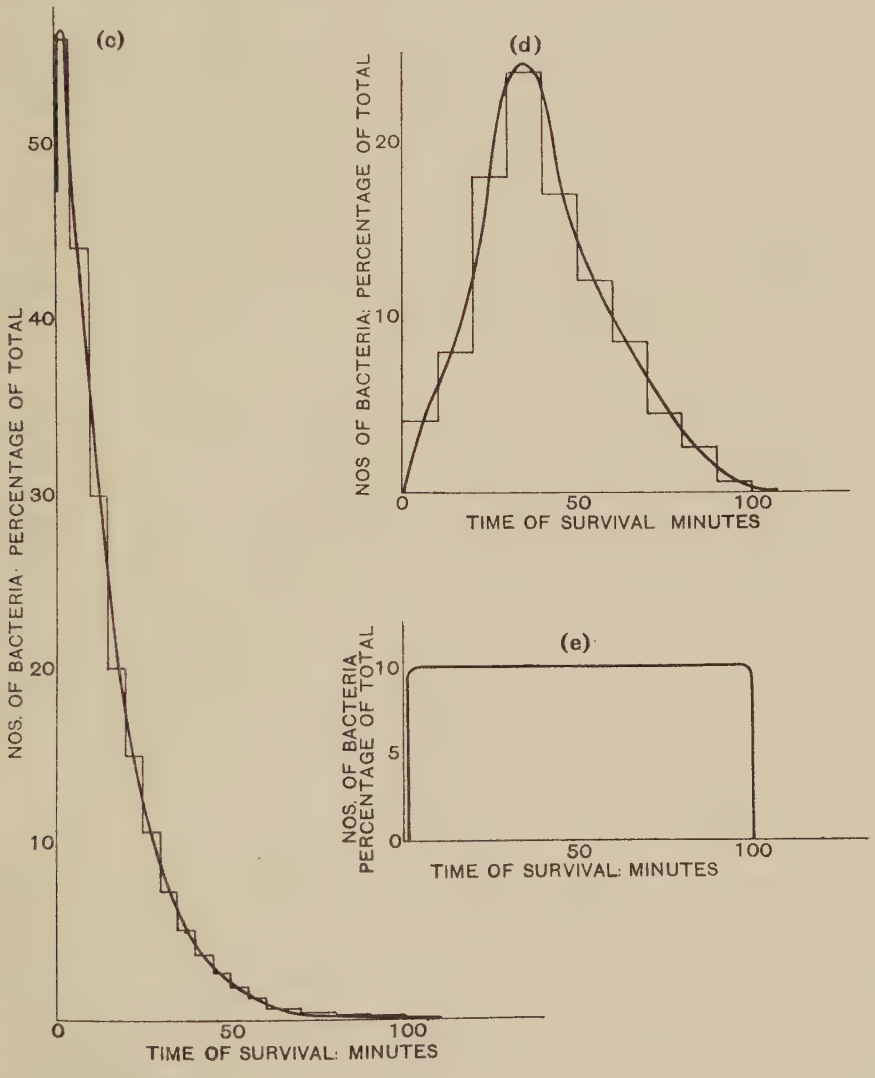

FIG. 2—*continued.*

(c), (d), (e) Curves showing frequency distribution of ' biological resistances '.

(c) Derived from logarithmic curve A.

(d) Derived from the sigmoid curve B.

(e) If the different groups containing bacteria of uniform resistances contain equal numbers of cells.

Abscissæ = ' resistance ' of cells expressed as survival times. Ordinates = numbers of cells in each group of differing ' resistance '.

In other cases the disinfection proceeds slowly at first and gradually becomes quicker during a subsequent period in which the majority of the bacterial population is killed ; there is then a period in which the small remainder succumbs at a comparatively slow rate (Curves B and B^1, Fig. 2 (b) and Fig. 3). Here the curves expressing the relation of survivors to time are of the sigmoid type. Disinfection of *B. coli* by heat (Eijkman,

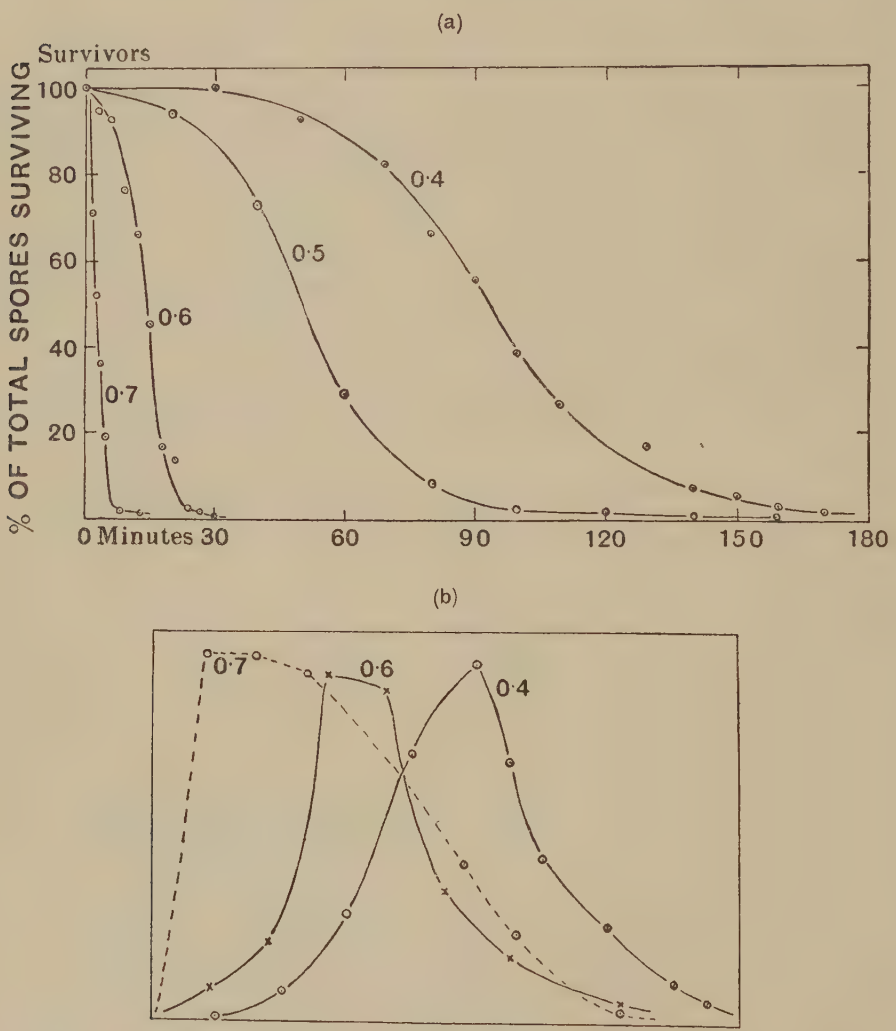

FIG. 3.—Disinfection of *Botrytis* spores with phenol, 0·4 to 0·7 per cent., at 25° C. (Henderson Smith, 1921). (a) Time-survivor curves. (b) Frequency curves derived from 0·4, 0·6 and 0·7 per cent. curves, showing increasing departure from normal with increasing concentration of phenol.

1908) and of *Staphylococcus* by phenol or heat (Chick, 1910) sometimes proceed in this way. Other examples are the disinfection by phenol or heat of yeast (' Rosahefe ', Eijkman, 1912) and of *Botrytis* spores (Smith, 1921,

1923), the killing of a ciliate protozoon *Colpidium* by mercuric chloride (Peters, 1920) and of tadpoles by dropping into hot water (Boycott, 1920).

Some organisms show an unexplained irregularity in the type of the survivor curve. While disinfection of *Staphylococcus* by hydrochloric acid or by drying at low temperatures proceeds logarithmically (Paul and his co-workers, 1909, 1910[1 & 2]), with both phenol and hot water time-survivor curves have been obtained which correspond to each of the three types described above (Chick, 1910). The same irregularity has been observed with *B. coli* by Eijkman (1908, 1912) and Falk and Winslow (1926), and with yeast cells by Fulmer and Buchanan (1923). It should be noted that in the experiments with hot water no regard was paid to the hydrogen-ion concentration of the solution. This factor has great influence on the course of the reaction in the heat coagulation of proteins (Chick and Martin, 1910, 1911, 1912), a process with which heat disinfection of bacteria shows a close analogy, and the fundamental experiments on the latter should be repeated with improved technique.

Theories advanced to explain Time-Survivor Relationships.

1. *Disinfection process considered as determined by varying biological resistance of individual bacteria.*

Disinfection processes showing survivor-time curves of the ' sigmoid ' type are what one would expect if the variation in survival times exhibited by the different individual bacteria were due to variations in what may be called ' inherent ', or permanent and irreversible, resistance to disinfection, these resistances being distributed among the bacterial population according to the usual arrangement of variable biological characteristics (Fig. 2, Curve d). In this scheme comparatively few bacteria would possess the minimal resistance and would be killed at the beginning of the process. The majority, possessing average resistance, would be killed at an intermediate rate, while a few, endowed with high resistance, would survive for a comparatively long time.

It is at first sight remarkable that disinfection should not *invariably* proceed in this manner, for it would be natural to expect the survival-time of individual bacteria to depend on their inherent resistance to the action of the disinfectant. But it is difficult, on any theory of varying biological resistance, to find a satisfactory explanation of the logarithmic relationship which is also frequently observed between the number of survivors and the time elapsing. Nevertheless, many bacteriologists, e.g. Hewlett (1909), Reichel (1909, 1923), Reichenbach (1911), Brooks (1918), Henderson Smith (1921, 1923), hold the view that different degrees of resistance to disinfectant action, due to normal biological variation, will account for the gradual nature of the disinfection process and for all the observed quantitative relations between survivors and times. Ponder (1924), arguing from analogy with the hæmolysis of red blood-cells, a process which frequently, though not universally, exhibits a sigmoid time-survivor curve, comes to the same conclusion.

This group of workers holds that a sigmoid time-survivor curve (B, B^1, Fig. 2) is characteristic of the disinfection process and the difficulty of explaining the numerous cases where the curve is logarithmic in character (A, A′, Fig. 2) is tackled in different ways by the different investigators. Seeing that, in those instances, the vast majority of the bacterial population succumbs during the *earlier* period of the operation, an explanation on the grounds of differing inherent resistances must assume that these resistances are distributed among the individual bacteria in a manner which is an exception to other studied instances of biological variation.

The difficulty has been well discussed by Brooks (1918). In his opinion the time-survivor curve in disinfection or hæmolysis is determined by two factors: (1) the nature of the ' fundamental reaction ' between the cell protoplasm and the disinfectant or hæmolysin, and (2) the varying resistance of individual cells to this action. If a cell's resistance be taken as proportional to its survival time, curves showing the distribution frequency of these resistances in a cell population can be constructed from the time-survivor curve. For processes where the latter is sigmoid in character, these frequency curves, while somewhat unsymmetrical and having a definite skew to the left [Curve (d), Fig. 2, and Curves (b), Fig. 3], resemble a type frequently obtained for distribution of variable biological characteristics. Brooks shows that, given such a distribution of resistances among the cells, the time-survivor curve will be of the sigmoid type, whether the ' fundamental reaction ' proceeds at a constant rate or is ' monomolecular ' in character.

Passing to disinfection processes where the time-survivor curve is of the logarithmic type, Brooks suggests the following two alternative explanations: (1) The ' fundamental reaction ' may be ' monomolecular ' and the ' resistances ' of the individual cells so arranged that the groups containing bacteria of different resistance contain equal numbers of cells [Curve (e), Fig. 2], in which case the time-survivor curve will be of similar form to that expressing the ' fundamental reaction '. There is, however, no precedent for such a distribution of a variable biological characteristic. (2) The ' fundamental reaction ' may proceed at constant rate and the resistances of the individual cells be arranged as in a logarithmic series. In this case the frequency distribution curve will be *entirely skewed* to the left, in fact, exponential in shape [Curve (c), Fig. 2], and the time-survivor curve would be determined entirely by this equally unconventional arrangement of resistances among the cells. The possibility that the resistance of the individual cells in a bacterial culture might be thus arranged has, however, been seriously considered by Reichenbach (1911), who bases his view on the peculiar way in which a bacterial population takes its origin. If certain assumptions are made, he shows that the distribution of ' biological ' resistance among the individuals of a bacterial culture might be according to almost any arrangement, and disinfection might proceed in a variety of ways, so that time-survivor curves might be sigmoid or logarithmic in type.

Henderson Smith (1921) found that disinfection of *Botrytis* spores was normally expressed by a sigmoid curve, but showed a tendency towards the logarithmic type when younger spores were used, or higher concentrations of disinfectant. By an ingenious process of reasoning, he shows that the latter type of survivor curve might still be the expression of variations in ' biological resistances ' arranged according to the usual rule, if it is assumed that the individual's survival time is proportional, not directly to this ' resistance ', but to its logarithm. For this hypothesis, however, which, though different from that of Reichenbach would be similar in result, no biological or experimental foundation has been advanced.

2. *The disinfection process considered analogous to a chemical reaction.*

Madsen and Nyman, Chick, Paul and his colleagues, Eijkman, Arrhenius and others, finding that disinfection of a *uniform* bacterial population proceeds in accordance with some rule analogous to the Mass Law obtaining in chemical reactions, consider that the process is capable of a similar interpretation. If, on the other hand, the cells do not possess uniform resistances, these will be distributed in the usual manner and the time-survivor curve will tend to become more or less sigmoid in character.

In a chemical reaction obeying the Mass Law, the reaction velocity (degree of change occurring in unit time) remains proportional to the concentration of reacting substances. In the case of disinfection the reagents are represented by the bacteria and the disinfectant. The disinfectant is usually present in so great an excess compared with the weight of bacteria acted upon, that its concentration may be regarded as constant throughout the operation. The rate of disinfection (reaction velocity) will therefore depend only upon the concentration of surviving bacteria, as in a bimolecular chemical reaction in which one reagent is present in large excess. Parallels are found in the hydrolysis of cane sugar and the heat denaturation of proteins. The union between the two sets of molecules (the sugar or protein on the one hand, and the water on the other) takes place as a unimolecular reaction, for, as ordinarily arranged, the dilute acid and the hot water, respectively, are present in so large an excess that their concentration remains constant, and the reaction velocity is determined only by the active mass of the second reagent, i.e. sugar and protein, respectively.

To explain these facts the chemist is forced to the belief that at any given moment during these reactions, *only a proportion of the sugar (or protein) molecules are in an active condition, able to undergo the union with water, and that this proportion bears a constant relation to the concentration of unchanged molecules.* This temporary susceptibility is believed to be due to cyclical differences in the internal energy of the molecule by virtue of which it is liable to attack in a certain phase only of its changing constitution. If a statistical view be taken of the happenings, this phase will appear to recur with precision and in obedience to some sort of rhythm, so that the *proportion* of vulnerable molecules remains constant throughout.

If such a conception be applied to disinfection, the individuals in a uniform bacterial population are assumed to be subject also to internal fluctuating changes, so that at any one moment a section only are liable to attack, the total number of vulnerable individuals being a constant proportion of the surviving population.

Experimental support for the idea that temporary changes of a rhythmic nature take place in the living bacterial cell can be found in some important observations of Eijkman (1912) which have received little notice. He sowed anthrax and other spores into nutrient broth, withdrew samples at intervals, subjected them to a temperature of 80° C. for a fixed period and transplanted to plate cultures. In this way, he determined the proportion of spores in the original sowing which remained ungerminated. From the results it was clear that the number of spores germinating in any small interval of time was proportional to the total number present, in other words, germination was proceeding in obedience to a Mass Law. With spores of *B. subtilis* the agreement was precise; with anthrax spores the survivor-curve showed a slight deviation towards the sigmoid type. Eijkman cites only three experiments, but the results are suggestive and should encourage more work along the same lines.

Discussion.

The view based on chemical analogy, in contradistinction to the theory of graded resistances, regards the logarithmic survivor-curve as expressing the least complicated disinfection processes and would refer the observed phenomena to the essential *similarity* of the individual bacteria. For this concept there is some experimental support. Any circumstances which might be supposed to increase uniformity among the bacteria used for the test have proved to increase the approximation to a simple mass relationship and not to disturb it. For example, the most accurate conformity with the logarithmic law was observed with anthrax and other spores which had been previously heated to from 70 to 80° C. By this procedure many weakly spores would perish and the resulting material be more uniform in resistance. Disinfection of 24 hours' cultures of *B. paratyphosus* usually proceeds more quickly at the beginning than if the rate were proportional only to the surviving population. Material obtained by repeated subculture after a few hours' growth, a device which may be expected to yield a more uniform collection of young, actively growing individuals, showed a nearer agreement with the Mass Law (Chick, 1908). A similar effect was noted by Henderson Smith (1921), when working with a collection of younger, and presumably more uniform, spores of *Botrytis*. More work along the lines suggested in this paragraph would be valuable.

It is possible that some further light might be shed upon the nature of the disinfection process by experiments in which the operation were interrupted during its course and the type of time-survivor curve observed when it was permitted to proceed again after an interval of time.

Disinfection by hot water would be most suitable for such an experiment, as the interruption could be made swiftly and completely by cooling, without risk of residual action during the interval. Convenient material would be *Botrytis* spores, where the time-survivor curve is of the sigmoid type (Smith, 1923), or *B. typhosus* or anthrax spores, where the death-rate is in accordance with the Mass Law. In the first case, where presumably the ' less resistant ' spores have already been killed, would the remainder behave as a more uniform population, exhibiting a curve more nearly logarithmic in shape and without a preliminary flat portion ? In the second case, where presumably a death process according to the Mass Law has been interrupted, will it be resumed in a similar manner ; or, on the other hand, will there be a preliminary period of lag, corresponding to the greater capacity to survive possessed by those individuals of ' greater resistance ', which were not killed off during the previous period of heating ?

Reichel (1909, 1923) and Reichenbach (1911) have brought forward a theoretical objection against adopting the Mass Law to explain death-rates of living organisms, which is difficult to meet. It is based on the discrepancy between the relative sizes of molecules and bacteria. In disinfection by heat, for example, the incapacity to propagate, which is adopted as the criterion of death, may be presumed to occur where a certain proportion of the molecules of the constituent proteins have been denaturated. One might, therefore, regard each separate organism as a separate solution of protein molecules in which this denaturation process was taking place according to the Mass Law. In similar bacteria one would expect that the same amount of damage would take place in the same time and all would ' die ' at the same moment. To explain the existing facts some further hypothesis is needed. One must assume that protein molecules in a living organism are not free protein molecules as in a solution of egg albumin, but are less independent one of another, and undergo their rhythmic changes not separately but in some way as a whole, so that at any moment of time, all or none are open to attack. This hypothesis as to the differences between ' dead ' protein and ' live ' protoplasm is, however, without experimental foundation.

Although the correct interpretation of the observed phenomena in disinfection is a matter of theoretical importance as promising an insight into the life-processes of the bacterial cell, the important matter from the practical point of view is to realize that the process is a gradual one and that in any collection of bacteria a minority will survive very much longer than the majority and on these attention must be focussed in order to obtain security from infection.

Mode of Action of Various Disinfectants.

Disinfectants appear to owe their toxic action upon living organisms to their capacity for reacting with protein and allied bodies, for example, by heat-denaturation and coagulation (hot water), by changes following the absorption of light of short wave-length (sunlight, ultra-violet light), by precipitation (phenol, cresol, formalin), by formation of bactericidal compounds (salts of mercury and other heavy metals, hypochlorites, and other chlorinating agents, and certain dyes), or by oxidation. Each class of disinfectant has special properties worthy of separate consideration.

Disinfection by the agency of heat or hot water shows a close analogy with the heat coagulation of proteins, not only in the time relations of the process, described above, but also (1) in the much greater resistance shown by bacteria to dry heat than to damp heat or hot water, (2) in the increase in disinfection rate caused by addition of acids and alkalis, and (3) in the unusually great increase in disinfection-rate caused by rise in temperature (see below, p. 199). The analogy is so close that one may conclude that, in heat-disinfection of bacteria, death is caused by heat-denaturation of the constituent proteins (Chick, 1910; Chick and Martin, 1910, 1911, 1912).

Sunlight and ultra-violet light. The effect upon solutions of pure proteins of sunlight, or of ultra-violet radiations from artificial sources, is similar to that of heat (Young, 1922; Clark, 1925), and the bactericidal effect of sunlight must be attributed to changes in bacterial protoplasm caused by photochemical action. Attempts to analyse the disinfectant action of sunlight by investigating the various portions of the sun's spectrum have shown the effect to be due to the ultra-violet radiations of short wave-length (Barnard and Morgan, 1903). Visible and near ultra-violet light, on the other hand, has a stimulating effect upon the growth of some fungi (Chavarria and Clark, 1924) and radiation of the long wave-length that can be transmitted through glass, has no influence on various bacteria at ordinary temperature, though some bactericidal action can be detected if temperature is raised and oxygen is present (Thiele and Wolf, 1907).

Browning and Russ (1917) investigated the action on *Staphylococcus*, *B. coli* and *B. typhosus* of the radiations from a tungsten arc and found that the limit of bactericidal action occurred at wave-length of 2,960 A.U. and extended to from 2,400 to 2,150 A.U., according to the organisms used and the length of exposure. The absorption limits of a suspension of *Staphylococcus* in a quartz vessel were also determined, and found to coincide exactly with the portion of the spectrum which possessed germicidal power. The fact that bactericidal action has not been demonstrated in radiations of wave-length longer than 2,960 A.U., indicates that the bactericidal action of sunlight is due to a very small section of the sun's radiation. Rays of 2,900 A.U. wave-length form the extreme limit of the ultra-violet radiation reaching the earth's surface from the sun, and,

except under conditions of great atmospheric clearness, radiations of shorter wave-length than 3,000 A.U. can rarely be detected. Increase of temperature, however, has great effect in increasing the bactericidal action of ultra-violet light (Thiele and Wolf), and it is possible that the coincident heat-rays from the sun may enhance very greatly the effect of the few effective light-rays.

Oxidation. Disinfectant action is possessed by such oxidizing agents as ozone (O_3), hydrogen peroxide (H_2O_2), permanganate of potash (K_2MnO_4), and it is possible that part of the germicidal potency of hypochlorites may be due to their oxidizing capacity. Presence of oxygen is an important factor in the disinfectant action which has been detected in the visible part of the sun's radiation (Wesbrook, 1896; Thiele and Wolf, 1907), and it is supposed that this is due to the formation of ozone or other active forms of oxygen. Paul and his colleagues (1910[1]) found that the death-rate of *Staphylococcus* by drying depended on the concentration of oxygen in the surrounding atmosphere, and their results suggest that death by this cause may be connected with some process of oxidation. They did not, however, make the crucial experiment of observing what happened when the operation of drying took place in an atmosphere of nitrogen or other indifferent gas.

Metallic salts. Metallic salts are precipitants for proteins and allied bodies, the protein acting as a weak acid and forming salt compounds in which the metal acts as positive radicle. These compounds prevent continued life and multiplication of the living cells. The halogen salts of mercury form a series whose germicidal potency is in the same order as the degree of electrolytic dissociation, thereby indicating the mercuric ion as the real disinfecting agent (Krönig and Paul, 1897). Any circumstance which tends to depress the degree of electrolytic dissociation, such as the addition of a neutral salt containing the same negative radicle (addition of NaCl to a solution of $HgCl_2$) is found to lower the germicidal action.

Mercuric chloride is the most potent among the salts of mercury and is one of the few disinfectants that can be relied upon for disinfection of anthrax and other spore-bearing organisms at ordinary temperatures (Koch, 1881). Its action, however, is slow and to some extent reversible. If a suspension of bacteria be added to a solution of mercuric chloride and centrifuged, the washed deposit will turn black on addition of a soluble sulphide, and if this treatment is not too long delayed, the bacteria will be found capable of multiplication when transferred to suitable nutrient media. Viable organisms of *Staphylococcus* have been recovered after immersion for 15 minutes in a 5 per cent. (saturated) solution of mercuric chloride (Chick and Martin, 1908[1]).

The double iodide of mercury and potassium (mercury biniodide) has had a great reputation in the past for sterilization of hands, instruments, catgut, &c., in surgical practice; this now appears to be without sufficient foundation (Bulloch, 1927). It is less irritating than mercuric chloride, but also much less effective.

Disinfection by mineral and other acids is partly due to the action of hydrogen ions, and in so far as this is true, the disinfecting action depends on the concentration of these ions (Krönig and Paul, 1897). The ' strong ' acids are the more powerful disinfectants, hydrochloric and sulphuric acids, for example, being more potent than acetic, when equimolecular solutions are compared. With some, as, for example, with hydrocyanic acid, the negative radicle has an additional toxic action.

Alkalis have been found to disinfect in proportion to the concentration of hydroxyl ions.

The rate of disinfection by *hot water* is greatly increased if the solution is made either acid or alkaline (Chick, 1910). This fact is applied in the practice of adding washing-soda to the water used for boiling surgical instruments, especially when these are suspected of contamination with spores such as those of tetanus or of the organisms associated with gas gangrene.

Chlorine, hypochlorites, chloramines. The disinfectant action of hypochlorites and other chlorinating agents depends on their capacity to yield up their chlorine to proteins and allied bodies. Chlorination of the protoplasm incorporated in bacteria and other living organisms appears to render it incapable of discharging its vital function. The other halogens possess an action similar to that of chlorine. The poisonous nature of bromine gas makes it unsuitable for use as a disinfectant, but iodine dissolved in potassium iodide is widely and satisfactorily employed as a skin disinfectant.

Disinfectants of this class are much reduced in value if other organic material is present with which the halogen can react. Nevertheless, chlorine and its compounds have been largely used in treatment of infected wounds, not only as hypochlorites (Dakin's solution), but also as chloramines, aromatic organic compounds in which the chlorine is attached to the nitrogen of an amido group. Chloramine T (paratoluene sodium sulphochloramide) investigated by Dakin and his colleagues (1915, 1916, 1917) is the best known example of this group. It is a stable, soluble, compound, less corrosive and less irritating than sodium hypochlorite, and has greater bactericidal power. It easily yields up chlorine to proteins and other cell constituents containing basic (NH_2) groups, and these chlorinated proteins themselves act in turn as antiseptics, and the action is thus carried on *in situ*.

Phenol, cresol and allied compounds. Phenol (carbolic acid, C_6H_5OH) is less soluble in water than in the protein-water phase of colloidal solutions of protein. The partition between the two phases takes place according to a fixed ratio, the protein phase taking up 3 to 16 times as much phenol as an equal weight of water, the figure depending upon the nature of the protein present (Reichel, 1909 ; Cooper, 1912). Up to a certain concentration of phenol this solvent action of the protein is reversible, and phenol is given up if extra water is added ; beyond this concentration, permanent precipitation of the protein takes place (Cooper, 1912). This

dual action explains many of the phenomena observed in disinfection by phenol. Presence of sodium chloride enhances greatly the disinfectant action of phenol (Spiro and Bruns, 1898 ; Reichel, 1909) and also influences the solubility partition coefficient in favour of the protein phase, the action in both cases depending on the concentration of salt. Presence of alcohol, or of oils and fats, influences the partition coefficient in the opposite direction, and depresses greatly the disinfectant action (Koch, 1881). With some vegetative organisms the depressing action of alcohol is masked by the disinfectant action of the alcohol *per se* (Cooper, 1912).

After solution of the phenol in the bacterial protoplasm, a precipitation of the latter takes place, and this must be regarded as the disinfection process. The comparatively large effect of temperature rise in increasing the rate of disinfection by phenol (Chick, 1908) is characteristic of reactions involving precipitation of proteins, while the effect of temperature on the solubility partition between water and protein is comparatively slight (Cooper, 1912). The effect of alteration in phenol concentration upon disinfection rate has been found to be as the fourth power of the concentrations employed, whereas the effect on the proportion dissolved would be in proportion to the concentration itself.

Influence of chemical constitution on germicidal value. Few satisfactory generalizations can be made regarding any connection between chemical constitution and germicidal value and among the selected facts given below many contradictions will be found.

Since the more important organic disinfectants are members of the aromatic series, it may be concluded that the benzene nucleus is important in this matter. The addition of the hydroxyl group in phenol changes benzene to an active germicide, and the further insertion of alkyl groups appears to increase the disinfectant action. In the coal-tar series, for example, the cresols (methyl phenol) and thymol (propyl-methyl-phenol) are more powerful germicides than phenol, and toluidine (methyl-amido-benzene) than aniline (amino-benzene) (Morgan and Cooper, 1912).

Substitution of the hydroxyl group by an amino group, as in aniline, lowers the disinfectant action of phenol, but the addition of amino-groups increases that of triphenylmethane and of acridine compounds (Browning and co-workers, 1913, 1918), and in the aliphatic series there are some amino compounds which are effective germicides, e.g. heptylamine (Morgan and Cooper, 1912).

In general, increase in molecular weight in a homologous series is accompanied by increase in germicidal power, e.g. in primary alcohols of the fatty series from methyl to octyl, and in the paraphenols from cresol to hexylphenol. Tilley and Schaffer (1926, 1927) have shown a regular numerical increase in disinfectant power to accompany the insertion of each fresh methyl group. With the quinone series, on the other hand, the germicidal value diminishes as the series is ascended (Morgan and Cooper, 1921). In the triphenylmethane compounds, insertion of alkyl groups in the amido groups (as in hexamethyl violet, malachite green, &c.) further

increases bactericidal action, but in compounds of the acridine series the opposite result is obtained (Browning and co-workers, 1913, 1922).

Influence of emulsification. The cresols and other tar acids obtained from distillation of coal are so sparingly soluble in water that they are frequently employed as emulsions, and these are found more effective disinfectants than solutions of equal strength. For example, an emulsion of tricresol was found by Rideal and Walker (1903–4) to be equivalent to a solution three times as concentrated. Chick and Martin (1908[2]) found that spores of *B. subtilis* were killed nearly 10 times as quickly by a $2\frac{1}{2}$ per cent. emulsion of tar acids in soap and water as by a solution of equal strength in alcohol.

This enhanced effect is due to adsorption of the emulsified particles on the surface of the bacterium, by which the concentration of the disinfectant is raised locally. If a bacterial suspension is added to such an emulsion, and the whole examined under the microscope on a dark background with side illumination (magnification of 1,100 diameters), the bacteria are seen to be continually bombarded by the small shining particles of disinfectant. Such a suspension, if compared with the same emulsified disinfectant to which no bacteria has been added, shows a great reduction in the number of these bodies, many having been appropriated by the bacteria. This can also be demonstrated by centrifuging the mixture, which, if the relative amounts of bacteria and tar acids are suitably arranged, will be cleared of all opacity.

This method was used by Chick and Martin (1908[2]) when investigating the amount of tar acids absorbed from emulsions of different known

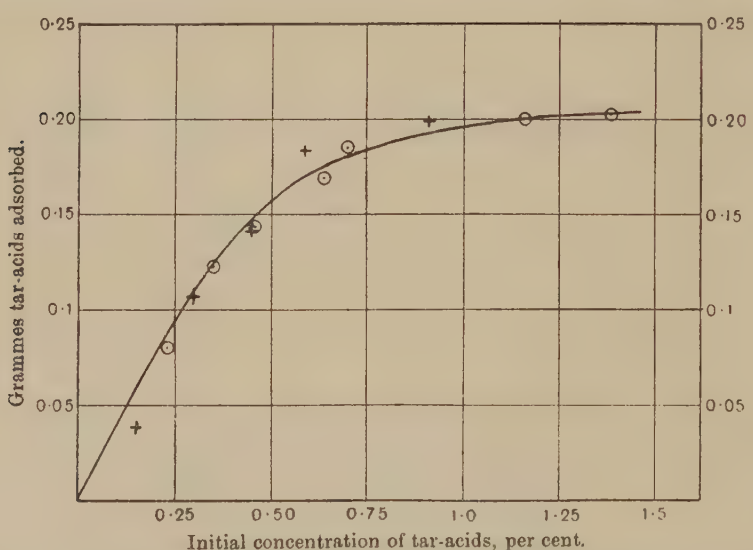

FIG. 4.—Adsorption of tar acids from an emulsified disinfectant solution by *Staphylococcus pyogenes aureus* and animal charcoal respectively. The circles represent the amounts of tar acids adsorbed by 1 gm. charcoal and the crosses that removed by 1 gm. staphylococcus in its natural condition.

strengths by a constant weight of bacteria. After remaining in contact for two hours, the mixtures were centrifuged and the residual tar acids in the supernatant liquor determined by extraction with ether. For lower concentrations of tar acids the amount removed was roughly proportional to the original concentration, but became relatively less as this concentration was increased and rapidly tended to a maximum. The results with bacteria were exactly similar to those obtained with animal charcoal, in fact, a curve drawn to show the absorption of charcoal would fit almost equally the results obtained with bacteria (Fig. 4).

The tendency shown by bacteria to absorb particles from an emulsion is a common property of most small bodies of organic origin. It follows that, in practice, the efficiency of emulsified disinfectants will be seriously deteriorated by the presence of any particulate organic matter in addition to the bacteria.

Influence of Change in Concentration of Disinfectant upon Disinfection Rate.

Change in concentration of disinfectant is accompanied in all cases by a corresponding change in the rate of disinfection. This relation, however, is different for different disinfectants. For example, 30 million *B. paratyphosus* were killed at 20° C. in $3\frac{1}{2}$ minutes by a solution of 12 per 1,000 phenol, while 240 minutes were required with 6 per 1,000 phenol (i.e. the rate was reduced to 1/70 when the concentration of the disinfectant was halved). With mercuric chloride at the same temperature, 30 million *B. paratyphosus* succumbed in 1·5 minutes in a concentration of 1 per 1,000, but the time of disinfection was increased only to 7 minutes, i.e. rate reduced only to 1/5 when the concentration of mercuric chloride was halved, and to 65 minutes when it was reduced to 1/100, namely, to 0·01 per 1,000 (Chick, 1908). A change in concentration of mercuric chloride from 1 to 0·01 per 1,000 corresponds to a reduction in concentration of mercuric ions of about one-third, so that, even if the mercuric ion be regarded as the real disinfecting agent, change of concentration has much less influence upon the rate of disinfection with mercuric chloride than, for example, with phenol. This point is of importance in devising methods for estimating germicidal power of disinfectants which shall not prove unfairly severe upon those belonging to the metallic salt category.

A logarithmic relation exists between the concentration of disinfectant and rate of disinfection which can be expressed by the following general formula :

$$\frac{C^n}{V} = \text{constant}$$

or $n \log C - \log V = \text{constant}$, where n is a constant depending on the nature of the disinfectant, C is the concentration of the disinfectant and V is the rate of disinfection. This relation, first discovered by Ikeda (1897)

TABLE II.

Influence of concentration of disinfectant upon rate of disinfection.

(*a*) Phenol, watery solution, and *Staphylococcus pyog. aur.*, 20° C. (dried in a film on the surface of garnets) (Gregerson, 1916).

Concentration of Phenol, % = C.	Time for complete Disinfection (minutes). *t.*	$C^4 \times t.$	4 log C + log *t*†.
0·5	300	18·3	1·26
0·625	90	13·7	1·14
0·75	75	23·7	1·37
0·875	30	17·6	1·24
1·0	20	20·0	1·30
1·25	8	19·5	1·29
1·5	3	15·2	1·18
1·75	1·5	14·1	1·15
2·0	1·0	16·0	1·20
2·5	0·375	14·6	1·17
3·0	0·187	15·2	1·18
		Average 17·1	

(*b*) Phenol, watery solution, and *B. paratyphosus*, 20° C. (Chick, 1908 and Watson, 1908).

Concentration of Phenol, % = C.	*Time for Disinfection (minutes) = *t.*	C^4 *t.*	$C^{5\cdot5}t.$	5·5 log C + log *t.*†
0·50	690	43·2	15·3	1·18
0·55	440	40·2	16·3	1·21
0·60	225	29·1	17·0	1·23
0·65	125	22·3	11·7	1·07
0·70	105	25·2	14·7	1·17
0·75	75	23·7	15·4	1·19
0·80	45	18·4	13·2	1·12
			Average 14·8	

* Reduction of 6 millions per c.cm. to less than 12 per c.cm.

† Calculated with logarithms to the base $_{10}$.

after examination of Krönig and Paul's results with anthrax spores and mercuric chloride, has since been confirmed by a series of workers using various disinfectants and different organisms.

Krönig and Paul determined the rate of disinfection by enumerating the surviving bacteria at successive intervals of time, but in the majority of experiments an end-point method has been used, in which an emulsion of bacteria in disinfectant of requisite concentration is sampled by withdrawing definite volumes from time to time, until these samples are found to contain no living bacteria demonstrable by subculture. The velocity of disinfection is then taken as inversely proportional to T, the ' time taken for disinfection ' and the above expression becomes

$$C^n t = \text{constant}$$

$$\text{or } n \log C + \log t = \text{constant}$$

The results of experiments with the half-dozen disinfectants which have been thus investigated are summarized in Table III. It will be seen that there is great divergence in the value of the exponent n for the same disinfectant, as determined from the results of different workers.

The highest value of n has been obtained for phenol, i.e. phenol is the disinfectant for which change of concentration makes most difference in disinfection rate. Watson (1908), using the experimental results of Chick (1908), found the value $n = 5 \cdot 5$ fitted experiments with *B. paratyphosus* and *Staphylococcus* [see Table II (*b*)]. Reichel (1909 II) and Gregerson (1916), working with *B. typhosus* and *Staphylococcus*, found the value 4 fitted their experiments [Table II (*a*)]. Reichel, however, in his calculation considered only the ' effective concentration ' of phenol, subtracting from the actual concentration the maximum which proved innocuous to the organism used. The details of the two experiments given in Table II show the degree of concordance which has been obtained in this type of work.

For reasons set out in the preceding section the action of phenol is considered to take place in two stages, the disinfectant being first dissolved in the bacterial protein, after which precipitation of the latter takes place. If disinfection rate were limited by the first process the influence of change in concentration of phenol would be that of a simple proportion ; the disproportionate effect observed must therefore be due to the influence of this change upon the protein precipitation, which must be regarded as the true disinfection process.

The formula $C^n t$ = constant fits the observed facts for disinfection of mercuric chloride if the exponent n is taken equal to 1, that is to say, rate of disinfection is proportional to the concentration of salt (Gregerson, 1916). If C is taken to express the concentration of mercuric ions the exponent is much greater, viz. : 4 to 5 (Watson, 1908).

In disinfection by hydrogen peroxide, the rate is proportional to the square root of the concentration, in other words the exponent in the above expression is equal to $\frac{1}{2}$ (Reichel, 1908, 1909[2]). Death-rate of

TABLE III.

Influence of Concentration of Disinfectant upon Disinfection Rate.

Disinfectant.	Method.	Organism.	Value of exponent n in expression $C^n t$ = const.	Observer.
Phenol . .	End-point, using suspensions of bacteria (experimental results of Chick, 1908).	*B. paratyphosus*	5·5	Watson, 1908.
		Staphylococcus aureus.	5·5	Watson, 1908.
	End-point	*B. typhosus* . .	4	Reichel, 1909.
		Staphylococcus	4	Reichel, 1909.
	End-point, using bacteria dried in films upon garnets.	*Staphylococcus*	4	Gregerson, 1916.
Thymol	End-point, using bacteria dried in films upon garnets.	*Staphylococcus*	4	Gregerson, 1916.
Mercuric chloride ($HgCl_2$)	Comparison of velocity constants, garnet method, results of Krönig and Paul (1897).	Anthrax spores	0·76 5·0	Ikeda, 1897.* Watson, 1908.*
	End-point, using suspensions.	*B. paratyphosus.*	3·0	Watson, 1908.
	End-point method, using garnets.	*Staphylococcus* and other vegetative organisms.	1·0	Gregerson, 1916.
	End-point method, using suspensions.	*Staphylococcus*	0·5	Gegenbauer, 1921.
Silver nitrate ($AgNO_3$)	End-point method, using suspensions.	*B. paratyphosus*	1·0	Watson, 1908.
Hydrochloric acid. (HCl)	Comparison of velocity constants, garnet method.	*Staphylococcus*	0·5	Paul Birstein and Reuss, 1910[2].
	End-point method with suspensions.	Anthrax . .	1·5	Gegenbauer and Reichel, 1913.
	End-point method with garnets.	*Staphylococcus*	1·0	Gregerson, 1916.
Hydrogen peroxide	End-point method with suspensions.	*B. typhosus* . .	0·5	Reichel, 1908 and 1909[2].
		Coli-like bacteria.	0·5	
Drying (action of Oxygen)	Comparison of velocity constants, using garnets.	*Staphylococcus*	0·5	Paul Birstein and Reuss, 1910[1].

* Ikeda used concentration of the salt in his calculation, and Watson, concentration of mercuric ions.

Staphylococcus by drying was found by Paul, Birstein and Reuss (1910[1]) also to be proportional to the square root of the concentration of oxygen in the vessel containing the dried films of bacteria (see above, p. 191).

Among the workers mentioned in the above paragraphs, Watson (1908) is the only one who has attempted to find any interpretation of the logarithmic relation existing between disinfection-rate and concentration of disinfectant. From analogy with chemical reactions obeying the mass law, he has hazarded the suggestion that the exponent n signifies the number of molecules of disinfectant which unite with one molecule of the reacting constituent in the bacterium. If this be so the reaction between phenol and bacterial protoplasm would be a reaction of the ' 5th order ', at least four molecules of the former with one molecule of the latter, and would be of a higher order than any reaction yet investigated in ordinary chemistry.

The observed relation in any one case, between rate of disinfection and change in concentration of disinfectant, has been used by some authorities for the standardization of disinfectants and to foretell, from the results obtained experimentally with one concentration, the effects which may be expected with some other, untested, concentration. A glance at Tables II and III, however, shows the risk of such procedure, seeing what great divergence exists between the results obtained by different workers using the same disinfectant and in some cases the same organism also. Until knowledge on this branch of the subject is much more certain, the value of a disinfectant can only be judged on the results of experiments devised to simulate as nearly as possible the conditions under which it is used in practice.

Influence of Temperature upon Disinfection Rate.

Robert Koch (1881) showed that the disinfection of anthrax spores with phenol was completed much more quickly if the temperature were raised. Madsen and Nyman (1907) found the same to be true, using mercuric chloride, and further showed that the rate of disinfection increased regularly with rise of temperature in fair accordance with the well-known equation of Arrhenius, the increase being about 2·5-fold for a 10° C. rise in temperature between 25 and 45° C. According to this law the effect of temperature is due to an increase in the energy possessed by the reacting molecules, and is proportional to the temperature as reckoned from absolute zero, —273° C. As temperature rises in arithmetical series, reaction rate increases in a geometrical ratio, in other words, there is a linear relation between the logarithm of disinfection rate and the temperature at which the process takes place. Table IV and Fig. 5 show the approximation to this law in the disinfection of *B. typhosus* by hot water at temperatures between 49 and 59° C.

Table V contains a summary of our present knowledge concerning influence of temperature on the action of disinfectants of different types. The extent to which disinfection rate is affected depends on the nature of

TABLE IV.

Temperature coefficient of disinfection of *B. typhosus* with hot water (end-point method).

	Temperature of Exp., ° C.	Time taken for disinfection,* minutes = t	$\text{Log}_{10} t$.	Log_{10} (temperature coefficient for 1° C.).	Temperature Coefficient	
					For 1° C. rise.	For 10° C. rise.
Exp. I ..	59·0	1·26	0·100			
	54·0	22·5	1·352	0·250	1·78	
	49·0	196·0	2·292	0·188	1·54	
Exp. II ..	57·0	7·5	0·875			
	52·1	53·0	1·724	0·173	1·46	
					Mean 1·60	110

* Reduction of about 5,000,000 per c.cm. to less than about 12 per c.cm.

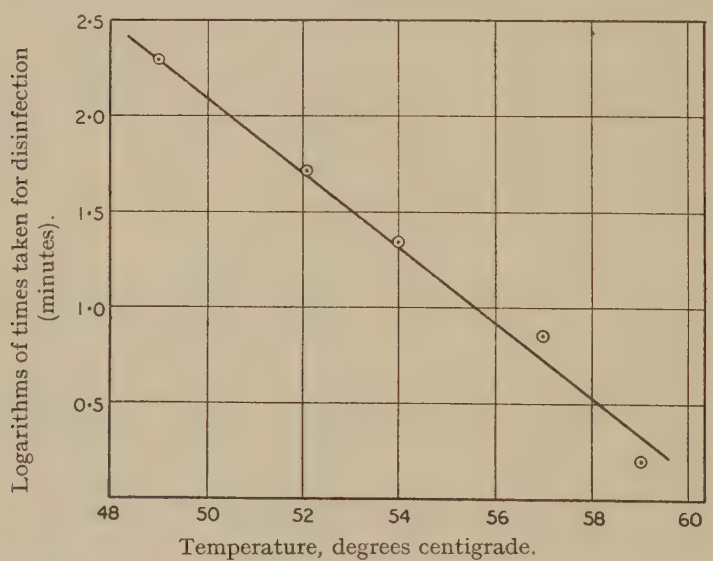

FIG. 5.—Effect of variation in temperature upon disinfection of *B. typhosus* by hot water (Table IV). (Chick, 1910.)

the process concerned in the germicidal action. Disinfection with metallic salts, which depends presumably upon a stoichiometric action between the metal and bacterial protein, is influenced to a degree comparable with other chemical reactions and is hastened about 2 to 3 times for 10° C. rise in temperature. With phenol and other coal-tar disinfectants the effect is much greater, disinfection rate being increased 5 to

Table V.
Influence of Temperature upon Disinfection Rate.

Agent.	Test Organism.	Temperature Coefficient		Range of Temperature ° C.	Method.	Observer.
		For 1° C. Rise.	For 10° C. Rise.			
Hot water	*B. typhosus* ..	1·60	110	49–54	End-point	Chick, 1910.
,, ...	*B. typhosus* ..	1·67	170	49–54	Comparison of velocity constants	,,
,, ...	*B. coli*.. ..	1·28	12	49–52	,, ,, ,, ,,	,,
,, ...	*Staphylococcus*	1·40	29	49–53	,, ,, ,, ,,	,,
,, ...	Anthrax spores		10	90–103	End-point	Ballner, 1902 (see Chick, 1908).
,, ...	Anthrax spores		40	80–90	Comparison of velocity constants	Eijkman, 1912.
,, ...	Foot-and-Mouth disease virus	1·7	201	50–60	,, ,, ,, ,,	Burbury, 1928.
,, ...	*Botrytis* spores	1·4 to 1·9	29 to 690*	31–50	Comparison of times taken to kill 50 per cent.	Smith, 1923.
Ultra-violet light ..	Hay infusoria..		4	11–21	End-point	Hill and Eidinow, 1923.
Drying	*Staphylococcus*		1·9 to 3·2*	1·7 to room temp.	Comparison of velocity constants	Paul and co-workers, 1909, 1910[1].
Hydrochloric acid ..	*Staphylococcus*		1·5 to 3·0*	1–47	,, ,, ,, ,,	Paul and co-workers, 1910[3]
Weak acid (buffered solution)	*B. typhosus* ..		1·6	0–30	,, ,, ,, ,,	Cohen, 1922.
	B. coli.. ..		3·4	0–30	,, ,, ,, ,,	,,
Mercuric chloride ($HgCl_2$)	*B. paratyphosus*		3 to 4	0–42	End-point	Chick, 1908.
	Anthrax spores		2·5	25–45	Comparison of velocity constants	Madsen and Nyman, 1907.
Silver nitrate ($AgNO_3$)	*B. paratyphosus*		3	15–40	End-point	Chick, 1908.
Phenol	*B. paratyphosus*		†7 to 15	0–35	,, ...	,,
,, ...	Anthrax spores		5·5	20–33	Comparison of velocity constants	,,
Emulsified tar acids ..	Anthrax spores		7 to 8·6	0–25	End-point	,,
Rabbit-serum	*B. coli*.. ..		2·4 to 3·3*	20–40	Comparison of velocity constants	Chick, 1913.
Goat-serum	*B. typhosus* ..		1·9	20–40	,, ,, ,, ,,	,,

* Coefficient varied with temperature zone. † Coefficient varied with concentration of disinfectant.

8 times for a similar rise. The influence of temperature rise upon disinfectation with hot water is even greater. With *B. typhosus* the rate of disinfection is increased 1·6 times if the temperature is raised 1° C., or 110 times (1·6 raised to the tenth power) if the temperature is raised 10° C. With *Staphylococcus* the temperature coefficient was 29, and with *B. coli*, 12, for a temperature rise of 10° C. The highest coefficient yet obtained, 1·7 for 1° C. or 201 for 10° C. rise, was found for the destruction by heat of the filterable virus of Foot-and-Mouth Disease. The actual figures obtained in these instances must, however, be regarded as approximate, since it is only possible to measure disinfection rate over a small range of temperature.

A high temperature coefficient is characteristic of many reactions involving proteins and protein-like bodies. In denaturation (coagulation) of proteins by hot water, for example, temperature coefficients of the order of 650 (egg-albumin, Chick and Martin, 1910) and 720 (alkaline wet hæmoglobin, Hartridge, 1912) have been determined for a 10° C. rise in temperature. The analogy here presented with heat disinfection of bacteria adds one more to those supporting the view that the latter is caused by heat denaturation of the bacterial protoplasm (see p. 190 above).

The effect of these high temperature coefficients is to give the impression that death of organisms (and coagulation of proteins) by heat takes place at a given temperature and suddenly, and the erroneous, though often useful, conception of a ' coagulation temperature ' for proteins and of a definite ' thermal death-point ' for different bacteria owes its origin to this circumstance. If, for example, it takes 20 minutes to kill 5 million *B. typhosus* at 55° C. it would take only 1 to 2 minutes at 60° C. and about 12 seconds at 65° C. If, then, the operation were being tested by withdrawal of one small sample after a short interval of time, say half a minute, the conclusion drawn would be that there was a ' thermal death-point ' for *B. typhosus* at a temperature between 60 and 65° C. It has needed careful quantitative investigation of the whole subject to elucidate this point, and to prove that temperature has no sudden or arbitrary effect, but that, as it is raised or lowered, it hastens or slows disinfection rate in a regular manner.

Antiseptic Dyes: Selective Antiseptics.

By C. H. Browning.

Many organic dyestuffs, especially those of basic type, are powerful antiseptics toward ordinary bacteria, but in general their bactericidal effect is produced slowly. It should be noted that the occurrence of staining of the bacteria by the dye does not indicate whether they are killed or not. A marked feature of some antiseptic dyes is their selective action upon one organism or group as compared with another. Certain dyes which are bactericidal only in the presence of light—photodynamic action (see Passow and Rimpau, 1924)—will not be considered here.

Influence of chemical and physical factors. Among basic dyes, i.e. compounds analogous to salts in which the organic dye-component acts as the positive (basic) radical, the triphenylmethane (see Browning, Gilmour and Gulbransen, 1918), acridine (see Browning, Cohen, Gaunt and Gulbransen, 1922), and anil quinoline series (Browning, Cohen, Ellingworth and Gulbransen, 1924, 1926, 1928), all contain members which are extremely potent antiseptics. In the triphenylmethane series the compounds with primary amino groups are much less active than those with tertiary amino groups ; thus parafuchsine and basic fuchsine are weak in their action compared with crystal violet and ethyl violet (gentian violet is a mixture). Similarly, Doebner's violet is inferior to malachite green and brilliant green. In the diamino acridine series, however, the tertiary amino compounds are not more active than those containing the corresponding primary amino groups. In other series, such as the anil quinolines, the relationships between constitution and antiseptic action are more complicated. As with antiseptics of other classes, the bactericidal power of dyes may depend greatly on the medium in which the organisms are suspended ; thus, in serum, as compared with water, the powerful action of members of the triphenylmethane series, e.g. brilliant green or crystal violet, is much reduced. On the other hand, certain antiseptics, e.g. those of the type of diamino acridine methochloride (acriflavine or typaflavine) and the anil quinolines, do not suffer reduction of activity in the presence of serum. The hydrogen-ion concentration of the medium may also have an important influence, e.g. the minimum concentration of acriflavine which kills *B. coli* in dilute peptone water of pH 4 to 5 is one hundred times greater than is required at pH 11 (Browning, Gulbransen and Kennaway, 1919 ; Eggerth, 1926). Acid organic dyes, in which the dye-component corresponds to the negative (acid) radical of a salt, are in general only weakly antiseptic, thus the conversion of an antiseptic basic dye into one of acid type by sulphonation, usually removes its activity ; similarly the sulphato triphenylmethane dyes (see Coplans and Green, 1927) corresponding to brilliant green and crystal violet are practically without antiseptic action. Certain antiseptic dyes are metallic compounds, e.g. the fluoresceine mercury derivative, mercurochrome-220 (Young, White and Swartz, 1919).

Time-rate of bactericidal action. The bactericidal action of dyes is in general exerted slowly as compared with that of antiseptics such as mercuric chloride or phenol. Further, concentrations of the antiseptic dyes which ultimately kill the organisms at first merely inhibit their growth ; this property of interfering with proliferation has been termed ' bacteriostatic action ' or ' genesistasis ' (Churchman, 1923). Accordingly, in order to estimate their potency it is necessary that such antiseptics should remain in contact with the bacteria for a long period. Of course, it is essential that the medium in which the organisms are suspended should not by itself be harmful to their vitality, as may be the case with water or saline. This may be avoided by incorporating varying concentrations

of the antiseptic either in solid or liquid nutrient medium, and then inoculating with a constant amount of organisms. After sufficient time for action has been allowed, say up to 48 hours at 37° C., the lowest concentration of the substance which has proved lethal is noted. When fluid medium is used and the amount of organisms employed as inoculum is insufficient to impart any obvious turbidity, it is often found that the minimum concentration of the substance which is lethal, as shown by the absence of growth in subculture, is considerably greater than that which suffices merely to inhibit growth. In the latter case the medium containing the antiseptic remains transparent after inoculation but yields a growth on subculture. It is noteworthy that in repeated experiments the minimum inhibitory concentration tends to be more constant than the bactericidal. In the case of virulent bacteria, the organisms after treatment may also be injected into a susceptible animal, since though not killed they may have been deprived of pathogenic action.

Selective action. The susceptibility of organisms such as staphylococci, diphtheroids and aerobic sporing bacilli, e.g. *B. anthracis*, and their spores to the antiseptic action of gentian violet or crystal violet, and, in contrast, the comparative resistance of *B. coli* to these substances was repeatedly noted and was related to the behaviour of the respective organisms when stained by Gram's method. The examination by Churchman (1912, 1913, 1921, 1923) and others of a large series of bacteria has established the general correspondence between Gram-positiveness and susceptibility to these dyes, on the one hand, and Gram-negativeness and resistance on the other (see Vol. IX). The rule, however, is not invariable, since certain Gram-negative organisms such as *V. choleræ* and the Gram-negative cocci are susceptible ; again, streptococci are more resistant than staphylococci (Krumwiede and Pratt, 1914). Also, certain strains of *B. enteritidis* are susceptible, although the majority are resistant (Churchman and Michael, 1912). Other classes of antiseptic dyes also show this difference in behaviour towards staphylococcus and *B. coli*, e.g. the cyanines, but many of the acridine and the anil quinoline compounds act on both organisms to a practically equal degree in neutral or slightly alkaline media. Acid fuchsine in contrast to the basic triphenylmethane dyes is more harmful to Gram-negative than to Gram-positive bacilli, especially at 45° C. (Churchman, 1923). Within a group of related organisms also, selective action may be manifested ; thus, most strains of *B. typhosus* are relatively insusceptible to brilliant green as compared with common intestinal types of coliform bacilli (Drigalski and Conradi, 1902). The paratyphoid bacilli exhibit high resistance to this dye also ; in addition, there are highly resistant organisms of coliform and other species (see Browning, 1918). Malachite green has a selective action similar to that of brilliant green, but less marked. There are many other examples of selective resistance (see Browning, Gilmour and Gulbransen, 1918), e.g. that of *C. diphtheriæ* and diphtheroids to telluric acid, as compared with staphylococci and certain coliform bacilli (Smith, 1918).

TABLE VI.

Substance.	Bactericidal concentration*			
	for *Staphylococcus aureus* in		for *B. coli communis* in	
	0·7 per cent. peptone water (faintly alkaline to litmus).	ox-serum (previously heated at 56° C. for 30 minutes).	0·7 per cent. peptone water (faintly alkaline to litmus).	ox-serum (previously heated at 56° C. for 30 minutes).
Acriflavine	1 : 200,000	1 : 200,000	1 : 20,000	1 : 100,000
Anil-48 [2 (Paradimethyl amino anil)—6· methyl quinoline methochloride].	1 : 200,000	1 : 200,000	1 : 200,000	1 : 200,000
Brilliant green	1 : 10,000,000	1 : 20,000	1 : 130,000	1 : 3,500
Crystal violet	1 : 4,000,000	1 : 400,000	1 : 8,000	1 : 8,000
Mercurochrome-220	1 : 100,000	1 : 1,000	1 : 100,000	1 : 1,000
Parafuchsine	1 : 100,000	1 : 20,000	(1 : 1,000 fails)	1 : 2,000

* 1 c.cm. of medium containing varying concentrations of the substances was inoculated with 0·1 c.cm. of a 1 : 1,000 dilution of a 24-hours' peptone-water culture : the *bactericidal concentration* = the lowest concentration which killed the organisms, as tested by subculture, after the mixtures had been kept at 37° C. for 48 hours.

Clearly, selective antiseptic action must depend on physico-chemical differences between the organisms, the exact nature of which, however, is unknown.

Acquired resistance. Shiga (1913), by growing *V. choleræ* in medium containing a sublethal concentration of acriflavine (trypaflavine) or other dyes and then subculturing into progressively higher concentrations, succeeded in developing dye-resistant strains of the organism. The resistance exhibited distinct specificity, but there was also some increase of resistance to other substances, especially those chemically related. Similar results have been recorded with various bacteria. The writer's own observations with *Staphylococcus aureus* and *B. coli* suggest, however, that it is not easy in all cases to obtain resistant strains (Browning and Gulbransen, unpublished ; see also Meader and Feirer, 1926).

Table VI (p. 205) illustrates results obtained with some representative antiseptic dyes.

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CHAPTER VI. THE METABOLISM OF BACTERIA.

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General Conditions of Nutrition.

The bacteria, in common with the rest of the *Fungi*, along with which they are usually classified, are not provided with chlorophyll and are unable to obtain their carbon from carbonic acid by the aid of light. They are as a rule dependent for this element on preformed carbon compounds of greater complexity than this, and these compounds are usually such that they are either directly assimilable or are converted by oxidation or by fermentative decomposition into assimilable compounds. In some cases, however, bacteria are capable of obtaining their carbon from compounds which require energy for their decomposition, such as carbon dioxide itself, the conversion of which into glucose, for example, entails an absorption of 672 Cals. per gram-molecule of glucose formed. In these cases the necessary supply of energy is obtained from some

$$6\,CO_2 + 6\,H_2O = C_6H_{12}O_6 + 6\,O_2 - 672\text{ Cal.}$$

concomitant change. A striking example of this is afforded by the nitrosifying and nitrifying bacteria, which can utilize the carbon of carbonic acid, but only when they are respectively simultaneously oxidizing ammonia to nitrous acid or nitrous to nitric acid, reactions which yield a considerable supply of energy.

$$NH_3 + 3\,O = H_2O + HNO_2 + 78{\cdot}8\text{ Cal.}$$
$$HNO_2 + O = HNO_3 + 18{\cdot}3\text{ Cal.}$$

The relation between the amounts of ammonia or nitrous acid oxidized and of carbon dioxide assimilated is a quantitative one, and a ratio can be experimentally determined between these two quantities (Winogradsky, 1890). Somewhat similar conditions exist among the 'sulphur' and 'iron' bacteria, which utilize the oxidation of sulphur and ferrous hydroxide respectively as sources of energy.

The relations of bacteria to nitrogen are closely analogous to those in which they stand to carbon. The great majority of organisms can satisfy their requirements of nitrogen from more or less complex compounds of C, H and N, such as the amino-acids, peptides, peptones and albumoses derived from proteins by hydrolysis, amides of organic acids, &c. In addition, a large number of organisms can, in presence of a suitable source of carbon, assimilate the nitrogen of such a simple compound as ammonia, NH_3. Only a small number of organisms can utilize nitrates as a source of nitrogen, although these constitute the best

source for the higher plants. A case of special interest is the utilization of atmospheric nitrogen by many soil organisms, such as *Azotobacter*. This only appears to be possible when a supply of energy is provided by the other constituents of the medium. Thus, it has been shown for *Azotobacter* (Mockeridge, 1915) that there is a definite ratio between the amount of carbonaceous nutriment oxidized and the amount of nitrogen taken up; e.g. 1 gm. of mannitol provides sufficient energy for the fixation of about 12 mgm. of gaseous nitrogen.

The remaining elements of the bacterial organism, phosphorus, sulphur, chlorine, the metals, &c., can as a rule be supplied in the form of inorganic salts, but it seems probable that in the metabolism of sulphur, compounds containing —SH groups in inorganic or organic combination may play a special part.

Energy Requirements.

All living organisms require a supply of energy available for the chemical changes which are essential for the processes of renewal of cell material and for growth, and many organisms also require energy to enable them to perform external work of some kind or other. Throughout the animal and the greater part of the vegetable kingdoms this energy is supplied by processes ultimately involving the oxidation by atmospheric oxygen of material which has been originally ingested in the form of food, and conveyed, in a more or less altered form, to the cells in which oxidation occurs. In all these higher forms of life no permanent maintenance or growth can occur in the absence of oxygen, although a temporary 'oxygen debt' may be incurred in some cases, which is subsequently 'paid off' by increased oxygen consumption (see Davis and Slater, 1926, 1928). Among the bacteria, however, and in some other branches of the family of *Fungi* a mode of life exists which is independent of the presence of free oxygen. The source of energy in these cases is no longer oxidation by free oxygen, but the decomposition of complex substances into products the formation of which is accompanied by loss of energy, which thus becomes available for the necessities of the organism. In the production of lactic acid from sugar, for example, there is an evolution of heat amounting to about 24 Cals.

$$C_6H_{12}O_6 = 2\ C_3H_6O_3 + 24\ \text{Cals.}$$

This anaerobic life of bacteria was first observed by Pasteur, who perceived its relation to the process of fermentation and expressed it in his celebrated dictum 'Fermentation is life without air'. The same organism may have the power of following either of these lines of existence according to the conditions in which it is placed (facultative anaerobes), being able, for example, like *B. coli communis*, to obtain the necessary energy from glucose in the absence of oxygen, but requiring this element when the sole available source of energy is peptone. In other cases (obligatory anaerobes) the power of obtaining energy by the normal

process of oxidation is entirely wanting and growth can only take place in presence of materials which are fermentable by the organism in question. Oxygen, indeed, may be rapidly toxic to the organism.

Again, in some instances the energy may be obtained at the expense of oxygen present in the medium, not in the free state but combined. The bacteria bring about reduction of this compound and simultaneous oxidation of their food material, the nett result of the two reactions being a liberation of energy, available for the organism. A striking instance of this is afforded by *B. pyocyaneus* (Pakes and Jollyman, 1901), which is unable to grow in absence of free oxygen in a peptone broth medium. When potassium nitrate is added abundant growth occurs in the absence of free oxygen. At the same time the nitrate is reduced (nitrite being first formed and free nitrogen being ultimately evolved) and some (unknown) constituent of the medium is oxidized, thereby yielding a supply of energy.

Assuming that the nitrate is reduced to nitrite and that the oxidizable substance is lactic acid, which is converted into carbon dioxide and water, the energy changes would be approximately as follows :—

$$6\ KNO_3 = 6\ KNO_2 + 6\ O - 132\ Cal.$$
$$C_3H_6O_3 + 6\ O = 3\ CO_2 + 3\ H_2O + 324\ Cal.$$

The available energy is thus, $324 - 132 = 192$ Cal. To this class also belong systems of two compounds, one of which, in the presence of the organism but in the absence of free oxygen, is reduced and the other oxidized with formation of an assimilable compound (see Quastel and Wooldridge, 1929). Thus, *B. coli* will not grow anaerobically either on a lactate or a fumarate, but grows readily on a mixture of these, the fumarate being reduced to succinic acid and the lactate oxidized to pyruvic acid, which is assimilated. The whole change, moreover, yields a supply of energy :

$$\underset{\text{Lactic acid.}}{C_3H_6O_3} + \underset{\text{Fumaric acid.}}{C_4H_4O_4} = \underset{\text{Pyruvic acid (assimilable).}}{C_3H_4O_3} + \underset{\text{Succinic acid.}}{C_4H_6O_4} + 14\ Cal.$$

This subject is more fully discussed under the heading of anaerobiosis (p. 270).

Mineral Nutrition.

The mineral nutrition of bacteria has not been worked out with great accuracy as regards the necessity for traces of various elements. On the other hand, a certain number of elements must be present in measurable quantity to ensure luxuriant and continued growth, but these vary with different bacteria. The simplest mixture used by Braun and Cahn-Bronner (1921, 1922) in their experiments on *B. paratyphosus B* only contained (in addition to possible traces of other elements and sources of nitrogen and carbon) phosphorus and either sodium or potassium. Such a medium,

for example, contained 0·3 per cent. KH_2PO_4 as its sole mineral constituent. The growth was somewhat improved by the addition of NaCl (0·05 per cent.), but was also satisfactory when the potassium phosphate was entirely replaced by the corresponding sodium salt. In a basal solution containing 0·5 per cent. NaCl and 0·2 per cent. KH_2PO_4 neutralized with $NaHCO_3$ as mineral constituents, a number of bacteria grew well (*B. paratyphosus B, B. pyocyaneus, B. coli, Friedländer's bacillus V. cholerœ, B. enteritidis* Gärtner, *B. typhosus, B. fœcalis alkaligenes*). In some of these cases the addition of Mg, S and Ca is useful, and in others (e.g. the tubercle bacillus) Mg and S, in the form of sulphate, are essential (Braun *et al.*, 1924 ; Braun and Kondo, 1924). Chlorides appear to be in no case essential.

In addition to these constituents bacterial media, unless special precautions are taken, invariably contain traces of iron, silica and probably many other elements, about the necessity of which nothing is known. Traces of iron (Uschinsky, 1897 ; Lasseur, 1913) seem in some cases to assist growth. In this connection the stimulating effect produced by traces of manganese and zinc on the growth of certain moulds (Raulin, 1870 ; Bertrand and Javilier, 1912) may be remembered.

Sources of Nitrogen and Carbon.

Classification of Bacteria according to their Mode of Metabolism.

Bacteria have been classified in various ways with respect to their carbon and nitrogen requirements (see Fischer, 1903 ; Beijerinck, 1891). According to the convenient suggestion of Orla Jensen (1909) three classes may be distinguished.

1. Bacteria which can obtain both their carbon and nitrogen from inorganic sources (NH_3, HNO_3, N_2, CO_2, CO). These are termed *autotrophic*.

2. Bacteria which can obtain their nitrogen from inorganic sources but not their carbon.

3. Bacteria which cannot obtain either carbon or nitrogen from inorganic sources.

According to Fischer, *prototrophic* bacteria can assimilate elementary nitrogen. The remaining organisms are *metatrophic* and may be divided into three classes ; *autotrophic*, as above ; *heterotrophic* which require organic compounds for their nourishment ; *paratrophic*, which require complex substances of unknown composition found in the animal and vegetable organism : usually the substances required are of the nature of growth-promoting factors.

The Simplest Forms of Nutrition.

The simplest forms of nutrition exist among the bacteria of the soil. Some of them, as already stated, are capable of assimilating elementary nitrogen and utilizing it as the sole source of their nitrogenous constituents.

Other soil bacteria are capable of oxidizing hydrogen (Kaserer, 1905, 1906 ; Niklewski, 1908) CO_2 (Kaserer, 1906) or CH_4 (Kaserer, 1905 ; Söhngen, 1906), and thus derive energy which enables them to assimilate the nitrogen of ammonium salts and the carbon of carbon dioxide. In the latter case the CO_2 is probably reduced (by a feebly exothermic reaction) to formaldehyde, which is then assimilated (Kaserer)

$$H_2CO_3 + 4\,H = H{\cdot}CH : O + 2\,H_2O + 6\,\text{Cal.}$$

The simplest conditions for the continued growth of many bacteria belonging to Jensen's second class (which require organic carbon but can assimilate the nitrogen of simple components such as ammonia or in some cases nitrate) have been investigated by Braun in conjunction with Cahn-Bronner, Kondo and others (Braun and Cahn-Bronner, 1921, 1922 ; Braun and Kondo, 1924 ; Braun, Stamatelakis and Kondo, 1924 ; Braun, Stamatelakis, Kondo and Goldschmidt, 1924). The principle employed is that of ascertaining what carbon compounds are necessary for the utilization of various simple sources of nitrogen in presence of a sufficient supply of mineral matter. In this way two series of compounds are obtained, one of carbon compounds assimilable in presence of a definite source of nitrogen and the other of nitrogen compounds which are capable of supplying nitrogen to the organism in presence of a definite source of carbon. Frequently, however, as in the case of many amino-acids, the same compound can serve as the sole source of both nitrogen and carbon. Some of the results are presented in Table I. It will be seen that the bacteria used differ considerably in their power of assimilation. The organism, amongst those examined, which was found to be able to utilize the simplest materials was *B. pyocyaneus*, which, in presence of an ammonium salt can derive its carbon from such a simple source as formic acid and can also utilize acetic, lactic, malic, citric or succinic acid, arabinose or dextrose under the same conditions. The nutrient solutions in these cases are of remarkable simplicity, containing 0·5 per cent. of NaCl, 0·6 per cent. of ammonium phosphate and 0·4 per cent. of the sodium salt of the acid. On the simplest of all the organism is motile, produces its characteristic colouring matter and excretes a proteolytic enzyme. It can derive both carbon and nitrogen from alanine or aspartic acid, but not from glycine or leucine, although it can take its nitrogen from these in presence of an oxidizable source of carbon such as lactic acid. In presence of lactate it can also derive its nitrogen from a nitrate—a power possessed by few bacteria.

Braun and Cahn-Bronner at first thought that they had obtained evidence that this organism could grow on a solution of ammonium carbonate, but later (1922) are inclined to ascribe the minimal growth observed to the presence of nutrient material derived from the inoculum. The other bacteria investigated differ from *B. pyocyaneus* in many points of detail, evident from a study of Table I, none of them being able to assimilate the carbon of formic acid in presence of ammonia as source of

TABLE I.

	Formate.	Acetate.	Oxalate.	Lactate.	Succinate.	Malate.	Tartrate.	Citrate.	Glycerol.	Arabinose.	Dextrose.	Lactate. Nitrate.	Glycine.	Glycine. Lactate.	Alanine.	Alanine. Lactate.	Aspartate.	Aspartate. Lactate.	Leucine.	Leucine. Lactate.	Urea. Lactate.	Ethyl Alcohol. NH_3.	Alanine. Glycerol.	Glycerol. Nitrate.
	NH_3																							
B. pyocyaneus	+	+		+	+	+		+	+	+	+	+												
B. paratyphosus B ..	−	+	?	+	+	+	?	+	+	+	+	−	?	+	+	+	+	+	?	+	+	−		
B. typhosus	−	−	−	+	−	?	−	+	+	−		−	−	+	+	+	?	+	−	+	+			
B. paratyphosus A ..	−	−	−	+	−	?		+	+	−		?	−	+	+	+	?	+	−	+	?			
B. dysenteriæ		−	−	+	−	?		?	+	−		−	−	+	−	?	−	+	−	+				
B. coli		+	?	+	+	+	−	?	+	+	+	−	−	+	+	+	+	+	?	+	+			
B. fæcalis alkaligenes ..				+																				
B. tuberculosis		+		−	−	−			+		+				?							+	+	+
B. phlei		+		+					+		+				+							−		

nitrogen or, with the exception of *B. tuberculosis*, to utilize the nitrogen of nitrates. In some cases growth is very sparse and the organism may lose its characteristic motility—very few or no cilia being produced.

Some strains of bacteria, e.g. of *B. typhosus*, *B. paratyphosus B*, and *B. dysenteriæ*, are incapable of growing on these simple media.

Requirements for Aerobic and Anaerobic Growth.

Many of the organisms included in Table I are facultative anaerobes, and grow readily as anaerobes in 0·5 per cent. peptone and 1 per cent. dextrose. In no case, however, was anaerobic growth obtained on these simple media. Aerobic growth only is possible under these conditions, and in most cases the presence of gaseous oxygen is absolutely essential. Growth at the expense of the oxygen of a reducible compound can in some cases be realized. Thus, *B. pyocyaneus* grows in the absence of free oxygen in a medium containing a nitrate, an ammonium salt, aspartic acid and lactic acid, but not if the aspartic acid be omitted. In this case the energy necessary for the assimilation of the nitrogen of the aspartic acid is supplied by the oxidation of the lactic acid by the nitrate, which is itself reduced to nitrite (see p. 210).

B. paratyphosus B and the other facultative anaerobes of Table I will not grow anaerobically in this way, being unable to bring about the reaction between nitrate and lactic acid. In their case anaerobic growth was only attained by the addition of tryptophan (0·5 per cent.) to a medium containing 1 per cent. dextrose and 0·6 per cent. ammonium lactate. No other amino-acid produced the same effect, and the presence of dextrose was essential. Even those strains of *B. typhosus*, *B. paratyphosus B* and *B. dysenteriæ*, which are incapable of assimilating ammonia or the nitrogen of amino-acids such as alanine and aspartic acid from these simple solutions, grew anaerobically on this tryptophan medium.* In their case the energy necessary for the assimilation of the nitrogen of the tryptophan was provided by the fermentative decomposition of the glucose. The general conditions for anaerobic growth are discussed elsewhere (p. 270).

Mechanism of the Assimilation of the Nitrogen of Amino-acids by Bacteria.

Nothing is definitely known of the way in which the assimilation of the nitrogen of amino-acids is carried out by bacteria. The analogous case of yeast has, however, been carefully worked out by F. Ehrlich (1905; see Harden, 1923), and it is highly probable that something similar occurs with bacteria.

Assimilation of nitrogen by ordinary culture yeast from amino-acids only occurs when the necessary energy is supplied from some concomitant change such as the fermentation of a hexose or of pyruvic acid.

$$C_6H_{12}O_6 = 2\ C_2H_6O + 2\ CO_2 + 22\ \text{Cal.}$$

* There is some doubt whether the tryptophan employed was pure (Braun and Cahn-Bronner, 1922), so that this effect may possibly have been due to the presence of some unknown impurity.

Certain ' wild yeasts ', which have greater oxidizing powers than culture yeast, can also assimilate the nitrogen of amino-acids in presence of lactic acid, glycerol and even of alcohol as sources of energy. The amino-group is removed from the amino-acid and taken up by the yeast and carbon dioxide is evolved. The remainder of the carbon residue of the amino-acid is not directly utilized by the yeast, but is found in the medium in the form of an alcohol (or sometimes an acid) containing one atom of carbon less than the original amino-acid :

$$R \cdot CH(NH_2) \cdot COOH + H_2O = R \cdot CH_2 \cdot OH + CO_2 + (NH_3).$$

Thus *iso*-leucine is converted into *iso*-amyl alcohol (a chief constituent of fusel oil) :

$$(CH_3)_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH + H_2O = (CH_3)_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot OH + CO_2 + (NH_3),$$

and the other amino-acids undergo similar changes.

The production of ammonia from proteins, amino-acids, &c., by bacteria is of very frequent occurrence (see pp. 241, 242), and it has been shown by Raistrick (1919), that in the presence of a utilizable source of carbon a large proportion of the nitrogen of these compounds is not liberated in the form of ammonia, but is assimilated by the organism, this action being, therefore, closely analogous to that which occurs with yeast.

It has already been pointed out that certain bacteria can obtain nitrogen, but not carbon, from some amino-acids, whereas in other cases both carbon and nitrogen can be supplied by a single amino-acid.

Assimilation of Carbon.

The exact form in which carbon is assimilated by bacteria is not definitely known. Presumably in the few organisms which are able to assimilate the carbon of carbon dioxide, the chemical changes are similar to those which occur in the higher plants. In the remaining organisms, by far the greater number, it seems probable that from the substrate is formed by a series of chemical changes, fermentation, oxidation and reduction, some substance capable of direct assimilation.

Quastel (1925, see also Quastel, Stephenson and Whetham, 1925) has suggested that for *B. coli communis* (and presumably for many other organisms) this assimilable compound is pyruvic acid, and he has shown experimentally that for this organism only substances which are at the same time capable (*a*) of being activated by *B. coli*, and (*b*) of yielding pyruvic acid with liberation of energy, are able to act as a nutrient carbon supply. Thus succinic acid, which fulfils these conditions, acts as a nutrient, maleic acid, which can give rise to pyruvic acid but is not activated by the bacillus, does not serve as a nutrient. Acetic acid is probably first converted into succinic acid. Formic acid although activated does not yield pyruvic acid and does not serve as a nutrient. It must be remembered, however, that formic acid acts as a nutrient for some bacteria (p. 213) so that the conditions of nutrition are not identical in all cases.

It follows from this conception that the function of fermentation is not only to provide energy but also to convert the substrate into a substance capable of being directly assimilated by the organism.

Usual Sources of Nitrogen and Carbon.

In the usual medium employed for the culture of pathogenic bacteria (peptone-bouillon) the sources of nitrogen are the extractives of meat (such as proteoses, creatine, creatinine, purine bases, carnosine, amino-acids, specially glutamic acid, phosphocarnic acid, &c.), and the more or less complex products of the hydrolysis of proteins added in the form of peptone (comprising polypeptides, true peptones and proteoses). All these compounds contain carbon and in addition there are present a number of non-nitrogenous carbon compounds, among which the most important are fats, glycogen, inositol and a number of organic acids, the chief of which are *d*-lactic acid, succinic acid and acetic acid. The trypsin-beef broth of Cole and Onslow (1916) contains all the above constituents, but a far larger proportion of amino-acids and peptides, formed by the digestion of the muscle proteins.

Proteins, both native and denatured, are utilizable by a considerable number of bacteria and are widely employed for the cultivation of anaerobic organisms (boiled meat medium), and, in the form of coagulated serum, for the culture of pathogenic organisms such as *Gonococcus*, *Pneumococcus*, *C. diphtheriæ*, &c.

On the other hand, it has been shown that carefully purified native proteins (egg-albumin, serum-proteins, edestin) are not attacked by a number of organisms, e.g. *B. coli* (Pfaundler, 1902), *Gonococcus*, &c., even including bacteria of strongly proteolytic powers such as *B. pyocyaneus*, *B. proteus vulgaris* (Bainbridge, 1911) and the anaerobic putrefactive organisms *B. putrificus*, *B. anthracis symptomatici* and *B. œdematis maligni* (Sperry and Rettger, 1915). Even when sufficient nitrogenous materials were present to permit of good growth, none of the aerobic organisms tested, with the exception of *B. proteus*, attacked the proteins (Bainbridge), whereas the anaerobes in the presence of mere traces of commercial peptone not only grew well but hydrolysed the protein.

The higher proteoses are also resistant to direct attack by many bacteria (Berman and Rettger 1918), and the availability of the nitrogen of many preparations of ' peptone ' is increased by more extensive digestion.

All these results seem to point to the conclusion that the permeability of the cell-wall of these bacteria is such that only comparatively simple products of protein hydrolysis can enter the cell and thus become available for assimilation.

The Fermentative Decomposition of Sugars and Allied Compounds.

Since the preparation in 1897 by Hans and Eduard Buchner of a cell-free extract from ground yeast which produced alcoholic fermentation of the sugars, Pasteur's conception of the essential connection of fermentative action with the life of the cell has been abandoned by the greater number of biochemists (see, however, Kostytschev and Faërmann, 1928; Schoen, 1928; Gotschlich, 1927; Nord and Franke, 1928). The various fermentations of sugars by moulds, yeasts and bacteria, in the course of which the carbon chain is broken and substances containing only 1, 2, 3 or 4 carbon atoms in their molecules are produced, are now usually regarded as the results of a co-ordinated series of chemical reactions accelerated by enzymes (termed desmolases), catalytic agents, which are produced by the living cell, but are capable of exerting their characteristic effects *in vitro*. The experimental realization of this conception is most complete for yeast, but in many cases it has been definitely shown that bacteria which have been treated in ways which are usually fatal to living cells still produce characteristic chemical changes. As regards the nature of these changes it has been proved beyond reasonable doubt that the dissimilation of a substance like sugar to form simple compounds such as lactic acid, carbon dioxide, alcohol, &c., is a process which takes place in a number of successive stages.

Alcoholic Fermentation.

As the process of fermentation has been most completely analysed in the case of the alcoholic fermentation produced by yeast, a brief account of the various changes involved may serve as an indication of what probably also takes place in bacterial fermentations.

The Production of a Reactive Compound containing Three Carbon Atoms.

This stage of the fermentation involves the rupture of the carbon chain, a process now termed *desmolysis*, and is probably identical not only in processes of fermentation by yeasts and bacteria, but also in the metabolism of sugar by higher plants and in the production of lactic acid in the muscle of vertebrates and invertebrates. The process requires the presence of a phosphate and is accompanied by the phosphorylation of some of the substrate with production of a phosphoric ester (Harden and Young, 1905; Iwanoff, 1907; Embden and Laquer, 1914; Meyerhof, 1926), but the exact sequence of events is not yet known. Possibly the introduction of a phosphoric group renders the molecule less stable, so that rupture occurs with formation of a 3-carbon compound, which is at once converted into other products, whilst a corresponding amount of stable phosphoric ester is also formed. Three of these esters have so far been isolated, fructosediphosphoric ester, a hexosemonophosphoric ester, which is a mixture of monophosphoric esters of glucose and fructose, and finally trehalosemonophosphoric ester hitherto only obtained by the use of dried yeast. It is, however, possible that the change is an example of a coupled

reaction, in which one change, in this case the rupture of the sugar molecule, is facilitated by the simultaneous occurrence of a second reaction, in this case the production of a phosphoric ester of a sugar. The fact that derivatives of both glucose and fructose are formed, whether the sugar used be glucose, fructose or mannose, shows that some modification of the sugar molecule precedes or accompanies the formation of the esters. In any case the resulting phosphoric esters are subsequently hydrolysed back into sugar and phosphoric acid, which re-enters the cycle and ensures the continuance of the reaction.

In the case of yeast preparations the nett result of this action may frequently be represented by the equations

(*a*) $2\,C_6H_{12}O_6 + 2\,Na_2HPO_4 = C_6H_{10}O_4(PO_4Na_2)_2 + 2\,H_2O + 2\,C_3H_6O_3$.

(*b*) $2\,C_3H_6O_3 = 2\,CO_2 + 2\,C_2H_6O$.

(*c*) $C_6H_{10}O_4(PO_4Na_2)_2 + 2\,H_2O = C_6H_{12}O_6 + 2\,Na_2HPO_4$.

In some cases, however, a considerable proportion of a hexosemonophosphoric acid is formed and the above equations no longer hold, although the ratio of the CO_2 evolved to the phosphorus esterified remains constant.

The enzyme concerned, Buchner's zymase, comprises two components, a non-dialysable, thermo-labile portion (apozymase) and a dialysable, thermo-stable portion (co-zymase), the presence of both being necessary for the action of the enzyme.

The Decomposition of the Three Carbon Compound.

This stage has been investigated very thoroughly by Neuberg (for literature, see Harden, 1923), who regards the three carbon compound as being methylglyoxal, $CH_3{\cdot}CO{\cdot}CHO$ or $CH_2{:}C(OH){\cdot}CHO$, an extremely labile substance which is readily formed by loss of water from the two trioses, dihydroxyacetone $CH_2(OH){\cdot}CO{\cdot}CH_2(OH)$, and glyceraldehyde $CH_2(OH){\cdot}CH(OH){\cdot}CHO$, and equally readily passes by intramolecular oxido-reduction into lactic acid, $CH_3{\cdot}CH(OH){\cdot}COOH$. According to Neuberg the further change of this substance proceeds by a series of dismutations, i.e. changes in which one molecule of a substance or a group present in a molecule, is reduced, whilst a second molecule, or group, is simultaneously oxidized, the hydrogen and oxygen being derived from a molecule of water. The simplest example is that of the conversion of acetaldehyde into alcohol (by reduction) and acetic acid (by oxidation), a change known as Cannizzaro's transformation, which is brought about *in vitro* by alkalis and *in vivo* by a special enzyme, dismutase, discovered in animal tissues by Parnas (1910) which is also present in yeast (Neuberg and Hirsch, 1919)

$$CH_3{\cdot}CHO + HOH + CH_3{\cdot}CHO = CH_3{\cdot}CH_2{\cdot}OH + CH_3{\cdot}COOH.$$

The conversion of methylgloxal into lactic acid is an instance of a similar change in which two groups situated in a single molecule are respectively oxidized and reduced.

$$CH_3{\cdot}CO{\cdot}CHO + H_2O = CH_3{\cdot}CH(OH){\cdot}COOH.$$

It can be brought about by means of alkalis and by the enzyme glyoxalase (Dakin and Dudley, 1913).

The series of changes which probably occurs in the conversion of methylgloxal into carbon dioxide and alcohol is as follows :

1. A portion of the methylgloxal is converted by a Cannizzaro transformation into glycerol and pyruvic acid

$$\begin{array}{rcl} CH_2:C(OH)\cdot CHO & & CH_2(OH)\cdot CH(OH)\cdot CH_2\cdot OH \\ & + 2\,H_2O = & + \\ CH_3\cdot CO\cdot CHO & & CH_3\cdot CO\cdot COOH. \end{array}$$

A small amount of glycerol is thus formed, as is actually found to be the case both with living yeast (Pasteur) and yeast preparations (Buchner), but this production of glycerol is not large, as once the chain of reactions is started it can proceed without the necessity of any further formation of glycerol.

2. The pyruvic acid is decomposed by the enzyme carboxylase (Neuberg and Hildesheimer, 1911)

$$CH_3\cdot CO\cdot COOH = CH_3\cdot CHO + CO_2$$

with formation of acetaldehyde and carbon dioxide.

3. The acetaldehyde and a molecule of methylgloxal then undergo a Cannizzaro transformation yielding alcohol and a molecule of pyruvic acid.

$$\begin{array}{rcl} CH_3\cdot CHO & H_2 & CH_3\cdot CH_2\cdot OH \\ + & \| & = \quad + \\ CH_3\cdot CO\cdot CHO & O & CH_2\cdot CO\cdot COOH. \end{array}$$

The latter then undergoes reaction 2 and the cycle (reactions 2 and 3) continues.

The Fixation Method.

The presence of acetaldehyde as a by-product of fermentation has long been known ; its formation as an intermediate product was proved by Neuberg and Reinfurth (1918) by the use of a ' fixation ' method—the principle of which has also been applied to many cases of bacterial fermentation. This consisted in adding to the fermenting mixture some substance capable of forming a stable, non-fermentable compound with acetaldehyde. The substance first used was sodium sulphite. In this way a stable compound of sodium bisulphite and acetaldehyde was formed

$$Na_2SO_3 + H_2O + C_2H_4O = NaHSO_3\cdot C_2H_4O + NaOH$$

and reaction 3 no longer occurred.

Under these circumstances the whole of the pyruvic acid was produced by reaction 1, and glycerol, from being a by-product, became one of the main products of the reaction, the nett change being represented by the equation :

$$\begin{array}{ccccccccccc} 2\,C_3H_6O_3 & = & C_3H_4O_3 & + & C_3H_8O_3 & = & CO_2 & + & C_2H_4O & + & C_3H_8O_3 \\ \text{Hydrate of methylglyoxal} & & \text{Pyruvic acid} & & \text{Glycerol} & & \text{Carbon dioxide} & & \text{Acetaldehyde} & & \text{Glycerol} \end{array}$$

As much as 68 per cent. of the theoretical amount of glycerol can be obtained in this way and the method has been used on the large scale.

The presence of pyruvic acid as an intermediate product was shown in a similar manner by the use of β-naphthylamine (v. Grab, 1921).

Bacterial Fermentations.

The origin of the various products of bacterial fermentation of the sugars, &c., has been much discussed. It seems probable that the early stages of the change are similar to those which occur in the case of yeast and yield a three-carbon compound (probably methylglyoxal) as primary product. The presence of a co-enzyme has been proved in many instances (Myrbäck and Euler, 1924; Virtanen, 1924, 1925[1 & 3]) and it is probable that this is identical with the co-zymase of alcoholic fermentation (Virtanen, 1925[3]). Phosphorylation of the substrate has been observed in a few instances, with lactic and propionic acid bacteria (Virtanen, 1924, 1925[1 & 2]), but nothing is known as to the nature of the phosphoric esters produced. It is not, however, excluded, and it is held by some workers, that the carbon chain of sugar may be split into groups containing 1, 2 or 4 carbon atoms.

The further changes undergone by the methylglyoxal depend on the specific nature of the organism and lead to much more varied products than are obtained from yeast.

(1) *Lactic acid* is formed by the action of the enzyme glyoxalase (Dakin and Dudley, 1913) on methylglyoxal.

$$CH_2{:}C(OH)\cdot CHO + H_2O = CH_3\cdot CH(OH)\cdot COOH.$$

Many bacteria have been shown to possess the power of converting methylglyoxal quantitatively, or almost quantitatively, into lactic acid (Neuberg and Simon, 1927; Neuberg and Gorr, 1925, 1926[1 & 2]) (see p. 222).

(2) *Pyruvic acid* has been detected as an intermediate product of bacterial fermentation by various observers, and, as it is not only fermented by a great number of organisms but serves as an assimilable source of carbon, it probably plays a very important part in bacterial nutrition (see p. 215). The change produced by bacteria is usually of a comparatively simple character, the acid being, for example, almost quantitatively decomposed into acetic acid, carbon dioxide and hydrogen

$$C_3H_4O_3 + H_2O = C_2H_4O_2 + CO_2 + H_2$$

by *B. coli* (de Graaf and Le Fèvre, 1925), and gas being produced from it by many other organisms which produce acid and gas from glucose (e.g. *B. paratyphosus B*, *B. enteritidis* Gärtner), but not by organisms such as *B. typhosus* and *B. dysenteriæ* which produce acid but no gas from glucose (Karczag and Moczár, 1913). The application of Neuberg's fixation method shows that acetaldehyde can be detected in all these cases. This would seem to show that the chemical change occurs in two stages

$$CH_3\cdot CO\cdot COOH = CH_3\cdot CHO + CO_2$$
$$CH_3\cdot CHO + H_2O = CH_3\cdot COOH + H_2$$

It is, however, possible that normally very little acetaldehyde is produced, the main reaction being :

(*a*) $CH_3 \cdot CO \cdot COOH + H_2O = CH_3 \cdot COOH + H \cdot COOH$

accompanied by (*b*) in the case of bacteria which decompose formic acid.

(*b*) $H \cdot COOH = CO_2 + H_2$.

Further work is necessary to decide this point. (For a discussion of this question, see Whetham, 1927.)

(3) *Acetaldehyde.* The application of the fixation method has shown (Neuberg and Nord, 1919 ; Neuberg and Arinstein, 1921 ; Peterson and Fred, 1920 ; Kumagawa, 1922) that acetaldehyde is a frequent intermediate product in these changes and it was actually found among the products of fermentation of sugar by *B. coli* by Grey (1913). Moreover, acetoin (or methylacetyl carbinol $CH_3 \cdot CH(OH) \cdot CO \cdot CH_3$) which is almost certainly formed by the condensation of two molecules of aldehyde, and may, therefore, be taken as a natural indicator of the intervention of this substance in the reaction (Whetham, 1927), has been shown (see Harden and Walpole, 1906; Harden and Norris, 1912[1 & 2]; and especially Lemoigne, 1919, 1923) to be a very usual product of bacterial action.

The origin of this acetaldehyde is still a debatable question. It may originate, as in the analogous case of yeast, from pyruvic acid (see p. 219)

$$CH_3 \cdot CO \cdot COOH = CH_3 \cdot CHO + CO_2,$$

or it may be formed directly from methylglyoxal along with formic acid, as is suggested for its origin in muscle by Lusk (1925)

$$CH_2 : C(OH) \cdot CHO + H_2O = CH_3 \cdot CHO + H \cdot COOH.$$

Whatever its origin, it is capable of yielding by simple reactions under biological conditions many of the final products of fermentation.

(*a*) Acetic acid and alcohol by dismutation (Cannizzaro's reaction)

$$2\ CH_3 \cdot CHO + H_2O = CH_3 \cdot COOH + CH_3 \cdot CH_2 \cdot OH.$$

This is effected by the enzyme dismutase discovered by Parnas (1910) in many animal tissues, and since found to occur in yeast and many bacteria (see p. 218).

(*b*) Alcohol by reduction

$$CH_3 \cdot CHO + 2\ H = CH_3 \cdot CH_2 \cdot OH.$$

(*c*) Acetylmethylcarbinol or acetoin by condensation of two molecules, effected by aid of an enzyme termed carboligase present in yeast and presumably many bacteria (Neuberg and Hirsch, 1921)

$$2\ CH_3 \cdot CHO = CH_3 \cdot CH(OH) \cdot CO \cdot CH_3.$$

Subsequent reduction converts this into butylene glycol

$$CH_3 \cdot CH(OH) \cdot CO \cdot CH_3 + 2\ H = CH_3 \cdot CH(OH) \cdot CH(OH) \cdot CH_3.$$

(*d*) Succinic acid is probably formed either directly from acetaldehyde by a combined condensation and oxidation

$$2\,CH_3\cdot CHO \rightarrow CHO\cdot CH_2\cdot CH_2\cdot CHO \rightarrow COOH\cdot CH_2\cdot CH_2\cdot COOH$$

or from acetic acid in an analogous manner (see Grey, 1917[1])

$$2\cdot CH_3\cdot COOH \rightarrow COOH\cdot CH_2\cdot CH_2\cdot COOH.$$

Various other origins have, however, been ascribed to succinic acid (see Scheffer, 1928).

(4) *Formic acid.* Formic acid is a very frequent product of bacterial action. It is probably formed directly from methylgloxal in the manner already indicated p. 221.

$$CH_2:C(OH)\cdot CHO + H_2O = CH_3\cdot CHO + H\cdot COOH$$

Bacteria which produce CO_2 and H_2 from sugars, &c., are also capable of decomposing formic acid and this substance is probably the chief source of the hydrogen evolved in bacterial fermentation.

(5 and 6) *Propionic* and *Butyric acid.* These are discussed later on pp. 227, 228.

Types of Bacterial Fermentation.

The various fermentations produced by bacteria are most easily classified on the basis of the chemical nature of the products. Such a classification, however, does not in general accord with the systematic classification of the bacteria, inasmuch as the bacteria of a single group may produce different types of fermentation. Some of the chief chemical types are the following.

The Lactic Acid Fermentation of the Sugars.

Lactic acid, $CH_3\cdot CH(OH)\cdot CO\cdot OH$, is a frequent product of bacterial fermentation of the sugars and higher alcohols. It probably in all cases owes its origin to the intermediate formation of a compound of the nature of methylglyoxal, which is then converted by the aid of a special enzyme (glyoxalase) into lactic acid

$$C_6H_{12}O_6 \rightarrow 2\,(CH_3\cdot CO\cdot CHO + H_2O) \rightarrow 2\,CH_3\cdot CH(OH)\cdot COOH.$$

This change has actually been demonstrated by direct experiment with a number of bacteria, including *B. coli* (Neuberg and Gorr, 1925[1]) *Lactobacillus, B. propionicus* (Neuberg and Gorr, 1926[1]), *B. lactis aerogenes* and *B. delbrücki* (Neuberg and Simon, 1927), inactive *dl*-lactic acid being in all cases produced in almost quantitative yield.

When the substrate is a sugar (or an alcohol) it is found that the proportion of lactic acid produced by various bacteria is very different, as are also its optical properties.

In some cases lactic acid is almost quantitatively produced from the hexoses, only traces of other substances being formed, and this represents the type of the pure lactic acid fermentation,

$$C_6H_{12}O_6 = 2\,C_3H_6O_3.$$

This is the case with many of the 'long' lactic acid bacteria, such as *B. bulgaricus* Leverson and Kühn, *B. delbrücki* Leichm., *B. acidificans longissimus* Lafar. According to Orla Jensen (1919), however, the quantitative production of lactic acid is not a characteristic of a definite group of bacteria, and his group of 'true lactic acid bacteria' includes many organisms which produce other substances as well as lactic acid from sugar. *B. bulgaricus* (Bertrand and Duchacek, 1909), for example, which ferments the natural hexoses and lactose, but not maltose, saccharose, mannitol and the pentoses, actually produced a total of about 0·36 gm. of acetic, succinic and formic acids from 20 gm. of glucose, the remainder being converted into optically inactive *dl*-lactic acid. *B. delbrücki*, on the other hand, although it produces exclusively lactic acid, produces the *l*-acid (Henneberg, 1909).

Further information on the very complex group of lactic acid bacteria may be obtained from the works of Orla Jensen (1919) and Henneberg (1909).

The Modified or Mixed Lactic Acid Fermentation.

A second very usual form of fermentation in which lactic acid is produced accompanied by a substantial proportion of other substances is characteristic of the organisms of the *B. coli* and *B. typhosus* groups. The nature and proportion of the products formed by some of these organisms have been investigated in great detail (see Pakes and Jollyman, 1901; Harden, 1901; Harden and Penfold, 1912; Grey, 1914, 1919[1 & 2], 1920, 1924, 1928; Grey and Young, 1921; Grimbert, 1901; Péré, 1892, 1896, 1898, and others). Although the opinion is still held by some (Goto, 1925; Neuberg and Gorr, 1926[2]) that the first product of the action of *B. coli* on sugar is lactic acid, the weight of the evidence (see especially Grey, 1919[2]) is in favour of the view that this organism and those of the group to which it belongs decompose their substrates, at all events after a certain intermediate stage in the decomposition has been reached, by independent enzymes which can be separately and differently affected by external factors, with the result that the proportions of the various products are not fixed but vary with the conditions. The actual products (apart from traces of acetaldehyde and other substances) consist of lactic acid, acetic acid, formic acid, succinic acid, alcohol, and in some cases gaseous carbon dioxide and hydrogen, and some organisms also produce methylacetylcarbinol (or acetoin, $CH_3 \cdot CH(OH) \cdot CO \cdot CH_3$) and butylene glycol, $CH_3 \cdot CH(OH) \cdot CH(OH) \cdot CH_3$. Some of the pentose sugars and the higher alcohols, in particular xylose, arabinose, mannitol, sorbitol and dulcitol are decomposed by some of the organisms with formation of the same products but in different proportions, the amount of alcohol, for example, being greatly increased when the substrate is an alcohol. The following table gives some typical figures.

TABLE II.

	Glucose.					Mannitol.
	B. coli in peptone water (Harden, 1901).	*B. coli* in mass inoculation (Grey, 1919[1]).	*B. coli* in yeast water (Scheffer, 1928).	*B. typhosus* in peptone water (Harden, 1901).	*B. dysenteriæ* Shiga-Kruse in peptone water (Scheffer, 1928).	*B. coli* in peptone water (Harden, 1901).
Lactic acid ..	40·1	20·0	42·4	49·5	68·4	18·6
Succinic acid ..	6·7	18·0	20·2	trace	7·4	8·9
Acetic acid ..	15·6	16·0	14·3	12·7	7·6	9·5
Alcohol	9·8	15·0	11·0	9·1	7·7	28·1
Formic acid ..	—	5·4	0·6	17·7	5·1	3·0
Carbon dioxide..	16·2	16·0	10·7	—	—	28·4
Hydrogen ..	0·7	0·35	0·5	—	—	1·5
Total ..	89·1	90·75	99·7	89·0	96·2	98·0
Ratio CO_2/H_2 ..	0·96	—	1·0	—	—	0·86

The optical character of the lactic acid is discussed on p. 248.

It is a characteristic of this type of fermentation that some organisms yield gas, in all cases a mixture of CO_2 and H_2, whereas others do not. This power of gas production is associated with that of decomposing formates into equal volumes of carbon dioxide and hydrogen (Pakes and Jollyman, 1901 ; Harden, 1901), and there seems little doubt that the origin of the gas in these cases is formic acid produced from the substrate. The gas evolved does not in all cases consist of a mixture of equal volumes of carbon dioxide and hydrogen, as in some instances the hydrogen appears to be used up in producing secondary reductions. Thus, organisms of the *B. lactis aerogenes* type, which produce large quantities of butylene glycol, a highly reduced product, yield a gas containing only 33 per cent. of hydrogen. When the gas is measured over a liquid medium, as in the diagnostic tests of Theobald Smith (1895), or in a Durham tube, the ratio of CO_2 to H_2 is disturbed by the superior solubility of the CO_2, so that the proportion of hydrogen present becomes greater. Thus the ratios of H_2/CO_2 were stated by Smith to be 2/1 for *B. coli* and 1/1 for *B. lactis aerogenes*, whereas after correction for the solubility of carbon dioxide these become about 1/1 and 0·5/1 respectively.

Three chief types of modified lactic acid fermentation may be distinguished.

1. *The type of fermentation produced by* B. coli (Table II).

The factors affecting the production of the various products have been investigated by Harden (1901, 1905), Harden and Penfold (1912), and especially by Grey and Grey and Young (1914–28). Anaerobic cultivation of the organism in peptone-water-glucose in presence of excess of chalk tends to the production of the type of result obtained by Harden, in which the ratio of CO_2 to H_2 is about 1, and alcohol and acetic acid are formed in approximately equal molecular proportions, whilst the lactic acid amounts to less than 50 per cent. of the sugar fermented and succinic acid to about 5 to 8 per cent. When, on the other hand, a large inoculation is made into a sugar solution containing no nitrogenous material, so that active growth is largely avoided (Grey, 1919[1]), less lactic acid is produced and more acetic acid, alcohol and succinic acid ; at the same time the ratio between alcohol and acetic acid is disturbed. A further modification is effected if the organism be first grown anaerobically (Grey and Young, 1921), still less lactic acid and very little succinic acid being then produced. Interesting light has been thrown on the nature of this fermentation by examining the products at intervals following a massive inoculation of a medium containing only sugar and mineral salts in presence of chalk (Grey, 1919[2], 1928). During the first 6 to 12 hours, the products consist almost exclusively of alcohol, acetic, succinic and formic acid, lactic acid being absent ; on this follows a period during which the chief product is lactic acid, whilst a considerable amount of the sugar is converted into a hydrolysable non-reducing carbohydrate. Subsequently this carbohydrate is again hydrolysed and fermented and further quantities of all the products are formed, including CO_2 and hydrogen. Grey, on the basis of counts of living organisms present at the various stages, considers that the production of the alcohol group of products is associated with the rapid death of a large proportion of the organisms which follows on inoculation, whereas lactic acid is produced during the proliferation of the surviving organisms which occurs during the second stage of the fermentation.

2. *The type of fermentation produced by* B. typhosus.

The chief, if not the only difference, between the action on the sugars, &c., by *B. typhosus* and *B. coli* respectively is that the former organism, being incapable of decomposing formic acid, does not produce carbonic acid and hydrogen. Its products accordingly (see Table II) contain a much higher proportion of formic acid than do those yielded by *B. coli*. A similar type of fermentation (see Table II) is produced by the various bacilli of dysentery (de Graaf and Le Fèvre, 1925 ; Scheffer, 1928).

3. *The type of fermentation produced by* B. lactis aerogenes *Esch.*

This organism, and two others, *B. cloacæ* Jordan and *B. capsulatus* Pfeiffer (see Durham, 1900–1 ; Howe, 1904 ; MacConkey, 1906) were early distinguished from *B. coli* by yielding a much lower proportion of

hydrogen to carbon dioxide (Smith, 1895), and by giving Voges and Proskauer's (1898) reaction (a fluorescent pink coloration when caustic soda is added to a culture in glucose-peptone-water). Examination of the products of the fermentation of sugar by *B. lactis aerogenes* (Harden and Walpole, 1906) and *B. cloacæ* (Thompson, 1912) showed that about 25 per cent. of the sugar had been converted into 2·3-butylene glycol, $CH_3 \cdot CH(OH) \cdot CH(OH) \cdot CH_3$, a small amount of acetylmethylcarbinol or acetoin, $CH_3 \cdot CH(OH) \cdot CO \cdot CH_3$ being also formed ; the remainder of the products were similar to those produced by *B. coli*, but the ratio of H_2/CO_2 (about 1 for *B. coli*) had fallen to about 0·5. Mannitol behaves in a similar manner, but only yields about half as much of the glycol as glucose. The following are typical results:

Table III.

	Per cent. of substance fermented.	
	Glucose.	Mannitol.
Alcohol	17·1	32·5
Acetic acid	5·1	2·5
Lactic acid	5·5	8·6
Succinic acid	2·4	3·2
Formic acid	1·0	1·5
Carbon dioxide	38·0	35·5
Acetoin	trace	trace
Butylene glycol	(25·0)	(12·5)
Hydrogen	0·74	1·24
H_2/CO_2	0·42	0·77

Voges and Proskauer's reaction is due (Harden, 1905) to the reaction of the acetylmethylcarbinol with the protein of the peptone solution (probably the arginine groups, see Harden and Norris, 1911) in alkaline solution in air. A similar reaction is given by diacetyl which is readily formed by oxidation. This reaction is given by a considerable number of bacteria, such as *B. subtilis*, *B. mesentericus*, *Tyrothrix tenuis* (see Desmots, 1904) and by *B. tartaricus* (see Grimbert, 1901, and especially Lemoigne, 1919, 1923) the first three of which do not give the type of fermentation characteristic of *B. lactis aerogenes*, but produce acetylmethylcarbinol and in some cases butylene glycol (Harden and Norris, 1912[2]).

The origin both of the acetylmethylcarbinol and of the butylene glycol is probably to be traced to acetaldehyde (p. 221) from which they can be derived by condensation,

$$CH_3 \cdot CHO + CHO \cdot CH_3 = CH_3 \cdot CH(OH) \cdot CO \cdot CH_3$$
$$CH_3 \cdot CHO + CHO \cdot CH_3 + 2\,H = CH_3 \cdot CH(OH) \cdot CH(OH) \cdot CH_3$$

accompanied in the case of the glycol by reduction. To this reducing action is due the deficiency of hydrogen among the fermentation products which is characteristic of this type of fermentation. The glycol has actually been obtained from acetaldehyde by the action of *B. lactis aerogenes* (Harden and Norris, 1912[1]) and by the action of yeast, the enzyme responsible for the synthesis being termed carboligase (Neuberg and Hirsch, 1921), and it is also formed from a number of other compounds.

4. A special case of some interest is *the 'mannitol' fermentation* which is brought about by an organism which occurs in wine. This organism ferments glucose and mannose completely to a mixture of acetic, lactic and carbonic acids and alcohol, but with fructose yields a large proportion of mannitol (60 to 70 per cent.), the remainder of the fructose being converted into acetic, lactic and carbonic acids, no alcohol being formed (Gayon and Dubourg, 1894, 1901; Mazé and Perrier, 1903).

The Propionic Acid Fermentation.

A characteristic fermentation of the simple sugars, glucose, fructose and galactose, resulting in the production of propionic and acetic acids, together with carbon dioxide, is effected by a number of strains of organisms grouped together as *B. acidi propionici* (von Freudenreich and Orla Jensen, 1907) which can be isolated from milk and cheese. The best source is Emmenthaler cheese (Gruyère), the 'eyes' in which are due to the formation of CO_2 by these bacteria. These organisms also produce propionic acid from lactic acid, pyruvic acid, glycerol and a number of organic acids.

In a medium consisting of yeast extract, and under anaerobic conditions, glycerol is converted almost quantitatively into propionic acid by these organisms

$$CH_2(OH)\cdot CH(OH)\cdot CH_2\cdot OH \rightarrow CH_3\cdot CH(OH)\cdot COOH,$$

whilst lactic acid yields a mixture of propionic acid, acetic acid and CO_2, approximately in accordance with the equation proposed by Fitz (1878)

$$3\,C_3H_6O_3 = 2\,C_3H_6O_2 + C_2H_4O_2 + CO_2 + H_2O$$

the molecular ratio of propionic acid to acetic acid actually realized being 1·8 instead of 2. Glucose and the other simple sugars yield the same products as lactic acid, but the proportion of acetic acid found is rather less, the molecular ratio (propionic acid)/(acetic acid) being greater than 2. Succinic acid is produced in all these fermentations, apparently from constituents of the medium (van Niel, 1928).

The chemistry of this mode of fermentation has been much discussed (see van Niel, 1928, where the literature is quoted). According to Virtanen 1925[2]) phosphorylation of the sugar occurs, as in yeast fermentation, and lactic acid is then formed and finally decomposed into propionic acid on the one hand and acetic acid and CO_2 on the other. Van Niel considers that methylglyoxal, $CH_3\cdot CO\cdot CHO$, is formed and that this is partly converted by a mixed process of oxidation and reduction to

propionic acid, and partly by oxidation into pyruvic acid, which then yields acetic acid and CO_2, and simultaneously reduces another molecule of methylglyoxal

$$2\,CH_3\cdot CO\cdot CHO + H_2O = CH_3\cdot CH_2\cdot COOH + CH_3\cdot CO\cdot COOH$$
$$CH_3\cdot CO\cdot COOH + CH_3\cdot CO\cdot CHO + H_2O = CH_3\cdot COOH + CO_2 + CH_3\cdot CH_2\cdot COOH.$$

Part of the propionic acid produced in the fermentation of glucose is probably formed by reduction of methylglyoxal at the expense of constituents of the medium, so that the ratio (propionic acid)/(acetic acid) becomes greater than 2.

The Butyric Fermentation of the Sugars.

The products of this action contain either *n*-butyric acid, $CH_3\cdot CH_2\cdot CH_2\cdot COOH$ or *n*-butyl alcohol, $CH_3\cdot CH_2\cdot CH_2\cdot CH_2\cdot OH$, or both and frequently also some of the higher homologues of butyric acid. The chemistry of this change has not been worked out on a strictly quantitative basis, but the same products, butyric acid, butyl alcohol, lactic, acetic and formic acids, alcohol, CO_2 and H_2 and traces of higher fatty acids are formed by a large number of bacteria, many of them anaerobes which occur in soil, whence they pass into the animal intestine, milk, &c., and some of which (e.g. *B. tetani*) are pathogenic (see Medical Research Committee, 1919).

In a typical butyric fermentation (Buchner and Meisenheimer, 1908; cf. Schattenfroh and Grassberger, 1900) in which the organism used was *B. butylicus*, Fitz, a spore-forming facultative anaerobe, there were obtained the following amounts of the chief products from 100 gm. of substrate.

	Glucose.	*Glycerol.*
n-Butyric acid	26·0	0·7
n-Butyl alcohol	0·7	19·6
Lactic acid	10	3·4

The chemistry of the process has attracted much attention inasmuch as the formation of butyl compounds from glycerol is accompanied by the synthesis of a carbon chain, and it has always been thought that this change is intimately connected with the mode of formation of the fatty acids present in the fats. Application of Neuberg's fixation method (see Neuberg and Arinstein, 1921, where the various theories are discussed) has shown that under these conditions acetaldehyde is produced (along with acetic acid and alcohol) and the formation of butyric acid and butyl alcohol inhibited. On the other hand, direct experiments indicate that the important intermediate product is not acetaldehyde, but the aldol formed by condensation of two molecules of pyruvic acid.

$$\begin{array}{ccccc} CH_3\cdot CO\cdot COOH & & CH_3\cdot C(OH)\cdot COOH & & CH_3\cdot CH_2\cdot CH_2\cdot COOH \\ & \rightarrow & | & \rightarrow & \\ CH_3\cdot CO\cdot COOH & & CH_2\cdot CO\cdot COOH & & + 2\,CO_2 \end{array}$$

which is readily converted by butyric bacteria into butyric acid. It seems probable then that pyruvic acid is produced (see p. 220) which is then converted into the aldol, which in its turn yields butyric acid, as shown above or, with simultaneous reduction, butyl alcohol

$$C_6H_8O_6 + 4\,H = 2\,CO_2 + H_2O + C_4H_{10}O.$$

The formation of higher homologues of butyric acid has been frequently observed (cf. Wolf and Telfer, 1917) and has been confirmed by fermenting a large amount of sugar in a synthetic medium, to avoid the production of these acids from the proteins of the medium (see p. 240). In this way about 1 per cent. of the sugar was obtained in the form of higher fatty acids, among which caproic acid, $C_6H_{12}O_2$ was identified (Neuberg and Arinstein, 1921).

Butyl alcohol-acetone fermentation. A special instance of the butyl fermentation is afforded by the action on starch of an organism belonging to the group of *B. amylobacter* (Bredemann, 1909). This organism converts starch almost quantitatively into butyl alcohol, acetone, carbon dioxide and hydrogen and has been used for the large scale production of the first two of these products. In the presence of chalk the products consist of butyric and acetic acids, along with carbon dioxide and hydrogen (for further information on this subject see Reilly, Hickenbottom, Henley and Thaysen, 1920; Speakman, 1920; Schoen, 1928[2]).

Acetone is also produced from carbohydrates, &c., by many other organisms (see Thaysen and Bunker, 1927).

The Methane Fermentation.

A comparatively small number of bacteria, some of them thermophilic, decompose cellulose and allied materials with evolution of methane. The first organism with this power was isolated by Omelianski from the mud of the river Neva (1895), and has been termed *B. methanigenes* Lehmann and Neumann. In a mineral medium containing an ammonium salt it decomposes pure cellulose with formation of about 6·8 per cent. of methane, 43 per cent. of carbon dioxide and 50 per cent. of fatty acids, consisting of about 9 molecular proportions of acetic and 1 of butyric acid. This corresponds to an evolution of about 95 c.cm. of methane per gm. of cellulose decomposed. No hydrogen is produced by this organism.

Methane is also formed by the decomposition of acetates by mixed cultures from sewage or manure (Hoppe-Seyler, 1876; Omelianski, 1906), and by an organism isolated by Mazé (1903).

This corresponds to the simple decomposition

$$CH_3{\cdot}COOH \rightarrow CH_4 + CO_2.$$

It is to organisms of this class that the production of methane in the natural decay of vegetable matter and in the alimentary tract of herbivorous and omnivorous animals is due.

The production of methane and the bacterial decomposition of cellulose are of great industrial importance and further information regarding them

may be obtained in Vol. III, p. 20, in Thaysen and Bunker's recent book on the subject (1927) and in an article by Omelianski (1905).

Fermentations by Oxidation and Reduction.

These are dealt with elsewhere (Chapter VII).

Application of Fermentation Reactions to Diagnostic Purposes.

The chemical action of bacteria on various substrates affords a rapid method of identification and is largely employed for this purpose. The use of these tests in the classification of bacteria is discussed elsewhere (p. 305). The chief classes of compounds which have been used in this way are given below, with the changes which they usually undergo in presence of bacteria.

1. *The simple sugars.* These comprise the hexoses, glucose, fructose, mannose and galactose ; the pentoses, arabinose and xylose, the methyl-pentose known as rhamnose or isodulcite $CH_3(CH \cdot OH)_4 \cdot CHO$, and occasionally tetroses (e.g. erythrose) and trioses (dihydroxyacetone and glyceraldehyde). If these substances are fermented they either yield acid alone or acid and gas. The type of fermentation is usually either the lactic or some form of the mixed lactic. Organisms which attack glucose usually also attack fructose and mannose, but this is not invariably the case. Thus *B. proteus, Micrococcus tetragenus* and *B. mesentericus* do not attack mannose although they all attack glucose (Kendall, Bly and Haner, 1923 ; Kendall and Yoshida, 1923).

2. *Di- and tri-saccharides.* It is probable that in all or nearly all cases of fermentation of di- and tri-saccharides these compounds are hydrolysed into the simple sugars of which they are condensation products, and these are then fermented. This is the case for yeasts (with the possible exception of maltose ; see Lindner, 1913), but the fact has not been so definitely established for bacteria. These compounds, therefore, as a rule provide a test for the presence or absence of an enzyme capable of hydrolysing the saccharide, provided that the organism is able to ferment one of the resulting products. The following is a list of the sugars usually employed with the products which they yield on hydrolysis, and the enzyme which accelerates the change.

	Saccharide.	*Enzyme.*	*Products of Hydrolysis.*
Di-saccharides ..	Maltose	Maltase	Glucose.
	Sucrose	Invertase (or sucrase).	Glucose and fructose.
	Lactose	Lactase	Galactose and glucose.
	Trehalose	Trehalase	Glucose.
	Cellobiose	Cellobiase	Glucose.
	Melibiose	Melibiase	Glucose and galactose.

	Saccharide.	*Enzyme.*	*Products of Hydrolysis.*
Tri-saccharide ..	Raffinose	Invertase and melibiase *or* 'emulsin' and invertase.	Fructose, galactose and glucose.

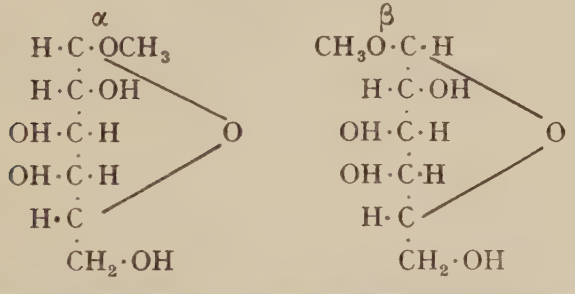
Raffinose presents the interesting feature that it is hydrolysed by invertase into fructose and melibiose, and by almond emulsin into galactose and sucrose.

3. *The polysaccharides.* Here again hydrolysis probably precedes fermentation. The chief substances used are starch, ' dextrin ', glycogen and inulin. Of these starch, dextrin and glycogen on complete hydrolysis yield glucose, whereas inulin yields fructose. ' Dextrin ' is an indefinite term applied to many of the intermediate products of starch hydrolysis. The material used for bacteriological purposes is usually the commercial dextrin prepared by heating starch with a trace of acid ; like starch it yields glucose on complete hydrolysis.

The diastases which attack starch also hydrolyse glycogen ; inulin requires a specific enzyme, inulase. The enzymic hydrolysis of all these compounds is, however, a very complex process and cannot further be considered here. According to Castellani (1927[1]), bacteria of the typhosus-dysentery group attack some starches but not others.

4. *Glucosides.* Glucosides, such as salicin, coniferin, &c., are sometimes employed. The glucosides are compounds formed by condensation between a molecule of a simple sugar and one of a hydroxy-compound, which may be itself an alcohol, yielding a heteroglucoside, or a sugar, yielding a homoglucoside. The reducing group of the sugar and a hydroxyl group of the second compound are involved in the condensation. Thus the methylglucosides are

Methylglucosides.



formed by the condensation of glucose and methyl alcohol and they exist in two stereo-isomeric forms, known as α- and β-glucosides. Compounds of similar constitution are formed by all the simple sugars and are known as fructosides, galactosides, &c. When the second compound is itself a

simple sugar the resulting homoglucoside is a disaccharide, thus maltose is glucose-α-glucoside. The α- and β-forms are hydrolysed by different highly specific enzymes. Thus α-methylglucoside is hydrolysed by maltase but not by emulsin, β-methylglucoside by emulsin but not by maltase. All natural plant heteroglucosides are β-glucosides and yield on hydrolysis by the appropriate enzyme (or by acid) the corresponding sugar and alcohol. A large number of these β-glucosides are hydrolysed by the enzyme preparation obtained from bitter almonds and usually known as emulsin. This is, however, a mixture of enzymes, one of which, termed β-phenolglucosidase, is responsible for the hydrolysis of many β-glucosides, such as phenolglucoside, salicin, helicin and probably arbutin. How far the hydrolysis of other glucosides is due to this same enzyme or to some other specific enzyme is not known (see on this subject, Oppenheimer, 1926, p. 275). As in the case of the saccharides it is probable, but not certainly proved, that the first action of bacteria on glucosides is to hydrolyse them, after which the resulting sugar is fermented. The glucosides, therefore, primarily serve as a test for the presence in the organism of the specific glucosidase required for their hydrolysis. Some of the chief glucosides used for bacteriological purposes are given below with their products of hydrolysis. Glucosides have been largely used to assist in the differentiation of Streptococci (Gordon, 1905 ; Andrewes and Horder, 1906 ; Holman, 1916 ; Kendall, Day, Walker and Ryan, 1919). Of the glucosides—salicin, amygdalin and arbutin—employed by Kendall *et al.*, salicin was fermented by all the 356 strains of *Streptococcus* tested, arbutin and amygdalin only by about 10 per cent. each. Similarly Gordon, and Andrews and Horder found that salicin was fermented by more strains than was coniferin.

Glucoside.	*Sugar.*	*Alcohol.*
Salicin	Glucose	Saligenin $OH \cdot C_6H_4 \cdot CH_2 \cdot OH$.
Helicin	Glucose	Salicylaldehyde $OH \cdot C_6H_4 \cdot CHO$.
Arbutin	Glucose	Quinol $C_6H_4(OH)_2$.
Amygdalin	Gentiobiose →glucose	Mandelonitril $C_6H_5 \cdot CH(OH) \cdot CN \rightarrow$ benzaldehyde and hydrocyanic acid $C_6H_5 \cdot CHO + HCN$.
Coniferin	Glucose	Coniferyl alcohol $C_6H_3(OH)(OCH_3) \cdot C_3H_4 \cdot OH$.

Populin yields glucose, benzoic acid and salicin and finally saligenin and benzoic acid.

The higher alcohols. The higher alcohols may be regarded as the reduction products of the simple sugars. Many of them have been employed for bacteriological purposes, the chief being glycerol $CH_2(OH) \cdot CH(OH) \cdot CH_2OH$, erythritol $CH_2(OH)[CH \cdot OH]_2CH_2OH$ adonitol and arabitol $CH_2(OH)[CH \cdot OH]_3 \cdot CH_2 \cdot OH$ and the hexitols $CH_2(OH) \cdot [CH \cdot OH]_4 \cdot CH_2 \cdot OH$, of which the stereo-isomeric compounds,

mannitol, dulcitol and sorbitol (the reduction products of mannose, galactose and glucose respectively) are usually employed.

Like the sugars, they yield either acid or acid and gas, undergoing fermentation of the mixed lactic type (see p. 223). Dulcitol is fermented by fewer organisms than either mannitol or sorbitol (Kendall, Day, Walker and Ryan, 1919). The last three differ from one another in the stereochemical configuration of the four hydroxyl groups. Bacteria show great specificity in their behaviour to compounds of this class. None of them is fermented by yeast.

Combined Effects of Fermentation by Two Species of Bacteria.

The general question of bacterial association, symbiosis or synergism is dealt with elsewhere (p. 156), the present remarks being limited to the results of fermentation in the presence of two species of bacteria.

The species of bacteria which produce typical fermentations with some of the hexoses, pentoses and higher alcohols may differ from one another with respect to their powers of fermenting individual members of these groups of compounds and the complex sugars. Thus, *B. typhosus*, *B. enteritidis* Gärtner, and many others do not attack lactose ; *B. Morgan No. 1* does not attack maltose, lactose, mannitol or sorbitol, and so on. In consequence of these properties results are sometimes obtained when two species of bacteria are present together which are not given by either species alone. Thus, *B. typhosus* which does not decompose formic acid into CO_2 and H_2 attacks mannitol with production of formic acid (Harden, 1901) ; *B. Morgan No. 1*, which produces acid and gas from glucose and, therefore, presumably decomposes formic acid, does not attack mannitol. When these two organisms are cultivated together in presence of mannitol acid and gas are produced, *B. Morgan No. 1* decomposing the formic acid produced by *B. typhosus*. A considerable number of cases of this kind have been recorded by Sears and Putnam (1923) and by Castellani (1926[1 & 2], 1927[1 & 2]) which are probably all explicable on these grounds. The whole question has also been discussed by Holman and Meekison (1926). The phenomenon has been used by Castellani as an aid to the diagnosis of the bacteria in question, especially those of the dysentery group, and as a bacteriological test for various sugars and alcohols ; but the method does not add anything to the information obtained by the use of individual organisms.

Fat Metabolism of Bacteria.

Decomposition of Fat by Bacteria.

The decomposition of fats by bacteria has been much discussed from various points of view, especially as to the participation of bacteria in the digestion of fats in the intestine. It was, however, early recognized (see Nencki, 1886) that they do not take any serious part in this change. It has been found (Sommarugo, 1894 ; Schreiber, 1902 ; Rubner, 1900) that a large number of bacteria decompose fats in the presence of oxygen and nutrient material, the action apparently being a primary simple

hydrolysis into fatty acid and glycerol, accompanied by further decomposition of the glycerol and oxidation of the fatty acid. Bacteria also take part, along with moulds and yeasts, in the production of rancidity in butter and other natural fats (see Orla Jensen, 1902). Eijkman (1901), who was confirmed by Söhngen (1911), showed that many bacteria excrete a lipase which hydrolyses fats and Söhngen also found that many bacteria thrive well when supplied with fat as exclusive source of carbon and ammonium chloride as source of nitrogen. Finally Wells and Corper (1912) demonstrated the existence of lipase in many bacteria which had been killed by toluene. They found that *S. pyogenes aureus*, *B. pyocyaneus*, *B. coli*, *B. dysenteriæ* and *B. tuberculosis* (in descending order of intensity) were all capable under these conditions of hydrolysing olive oil, triacetin and ethyl butyrate.

Formation of Fat by Bacteria.

The formation of fat is a regular feature of bacterial metabolism, the fat being stored in the cytoplasm where it can be detected (see Unna, 1896; Shattock, 1898; Dziergowski and Rekowski, 1895) by various reactions such as treatment with osmic acid, staining with Sudan III and other dyes, solubility in concentrated chloral hydrate solution and resistance to sodium hypochlorite solution (Meyer, 1901). The nature of the fatty material has been carefully investigated, especially in the case of the tubercle bacillus (see Vol. II), but comparatively little is known of the metabolic process by which it originates. The Timothy grass bacillus (*B. phlei* Möller), according to Stephenson and Whetham (1922), when supplied with mineral salts and ammonia under aerobic conditions, forms ether-soluble material most readily on a mixture of acetic acid and glucose, or acetic acid and lactic acid; less readily, but still freely, from glucose or lactic acid alone; while it grows well on all these media. With acetic acid alone scarcely any growth occurs. The maximum amount of ether-soluble material obtained was 42 mgm. per 100 c.cm. of medium, accompanied by the assimilation of 18 mgm. of nitrogen. When the glucose, &c., of the medium is exhausted, the amount of ether-soluble material slowly decreases.

The formation of fat by yeast which takes place under aerobic conditions from carbohydrates and many other compounds (see Smedley MacLean, 1922; Smedley MacLean and Hoffert, 1924, 1926; Smedley MacLean and Thomas, 1920; Daubney and Smedley MacLean, 1927) presents many analogies to the foregoing.

Production of Bacterial Gums.

Many bacteria produce in the liquid medium in which they are cultivated a soluble slimy or gummy product which frequently imparts to the fluid the property of forming viscid threads when poured from one vessel to another. The phenomenon is of some industrial importance as it occurs occasionally in milk (see Golding, 1912) and in beer (see Baker,

Day and Hulton, 1912) and other fermented liquids and is known as ' ropiness '. A large amount of investigation has been directed to the isolation of the organisms responsible for the change and to the conditions for the prevention of its occurrence, but until recently comparatively little was known of the chemical nature of the viscid materials. It was recognized that they might either contain or be free from nitrogen and that they were probably identical with, or closely related to, the material of which the capsule is composed. A large number of these substances has been isolated and described (see Buchanan, 1909 ; Lafar, 1905).

New interest has been aroused in these compounds since it has been discovered that they are in many cases serologically specific, yielding precipitates with high dilutions of the serum of an animal which has been injected with the organisms from which they are derived. The serological aspect of this question is treated elsewhere. Chemically the ' specific ' bacterial gums hitherto examined belong to several different types. The best known are those derived from the three serologically distinct types of *Pneumococcus*. Their chief properties are given in Table IV, quoted from Heidelberger (1927). Of these substances that from Type III has been most carefully studied (Heidelberger and Avery, 1923, 1924 ; Avery and Heidelberger, 1923, 1925 ; see also Heidelberger, 1927). This substance is a comparatively strong acid and can be purified by taking advantage of this fact. It is formed in broth cultures of the *Pneumococcus*, but the yield is greatly increased by adding glucose to the broth and then amounts to 35 to 40 gm. per 300 litres of medium. It is apparently a polysaccharide derivative formed by the condensation of an unknown number of units consisting of an aldobionic acid $C_{12}H_{20}O_{12}$, i.e. a compound of an aldose (probably glucose) with a hexoseuronic acid (of an analogous constitution to glycuronic acid, $CHO \cdot [CH(OH)]_4 \cdot COOH$). On hydrolysis of the polysaccharide derivative this aldobionic acid, which is difficult to hydrolyse, is liberated, together with a small amount of glucose, probably formed from it by partial hydrolysis. This acid forms a crystalline morphine salt, but has not itself been obtained crystalline. It has a reducing power of 50 per cent. of that of glucose and on boiling with acid slowly yields a small amount of glucose and some furfuraldehyde, probably derived from the hexoseuronic acid portion of the molecule.

The specific substance from Type II *Pneumococcus* yields on hydrolysis glucose equivalent to about 70 per cent. of its weight, the remaining products being unknown. That from Type I, on the other hand, contains nitrogen and is apparently at once a derivative of a galacturonic acid and an amino-sugar. A specific substance has also been obtained from the Friedländer bacillus (Type B) (see Table IV), which closely resembles both chemically and serologically that from Type II *Pneumococcus*, although the two are not serologically identical.

Specific substances of similar general character to the above have been obtained from a number of other organisms ; from the tubercle bacillus by Laidlaw and Dudley (1925) and by Mueller (1926), and from

TABLE IV.

Soluble specific substances of the three fixed antigenic types of pneumococcus and of Friedländer bacillus (Type B).

Type.	$[\alpha]_D$	Acid equivalent.	C	H	N	Reducing sugars on Hydrolysis.		Highest dilution giving precipitate with homologous immune serum.
						Calculated as glucose.		
I	+300°	—	43·3*	5·8	5·0†	28	(Galacturonic acid) (Amino-sugar derivative).	1 : 6,000,000
II	+ 74°	1,250	45·8	6·4	0·0	70	Glucose	1 : 5,000,000
III	− 33°	340	42·7	5·3	0·0	75	Aldobionic acid, glucose	1 : 6,000,000
Friedländer bacillus.	+100°	685	44·6	6·1	0·0	73	Glucose	1 : 2,000,000††

* Theory for $C_6H_{10}O_5$; C, 44·4 per cent.; H, 6·2 per cent.

† Amino-N, 2·5 per cent.

†† Rabbit antiserum.

another strain of Friedländer's bacillus by Mueller, Smith and Litarczek (1925), but their exact composition has not yet been ascertained.

It is to be noted that these substances are closely allied to the true gums and hemicelluloses, which are polysaccharide derivatives of acids, and to the pectins which contain the galacturonic acid residue.

Compounds of this kind are capable of a great range of differentiation in the structure of the carbohydrates and acids of the units of which they are composed as well as in their mode of combination and condensation.

It is not known whether the bacterial gums obtained by the earlier investigators (see Buchanan, 1909), the first of which was examined by Pasteur in 1861, are also serologically specific. Those free from nitrogen are generally regarded as condensation products of the simple sugars, termed glucosans, fructosans, galactans and arabans, which yield the corresponding simple sugars on hydrolysis, or in some cases are more complex and yield a mixture of sugars. Physically they are amorphous substances which form colloidal solutions in water, from which they are precipitated by alcohol. They do not reduce Fehling's solution.

The formation of gum by *B. radicicola* has been studied by Buchanan (1909) particularly with regard to the various sources from which it can be produced. He regards the substance as a glucosan, but did not examine the products of hydrolysis quantitatively. The most favourable sources are carbohydrates, in particular sucrose, glycerol, succinates, citrates, amygdalin and salicin. The nitrogen-containing 'gums' are formed by a great variety of organisms (see Rettger, 1903), and seem often to be of very complex structure.

Action of Bacteria on Nitrogenous Substances.

Putrefaction.

The spontaneous decomposition which meat and many other protein-containing materials undergo when exposed to air, and which is accompanied by the production of a characteristic unpleasant 'putrid' odour is usually termed putrefaction. The process is very complicated both bacteriologically and chemically, and results in a profound decomposition of the proteins present and the production of a large number of relatively simple compounds, among which fatty acids and bases predominate, and which may also comprise peptones, peptides, amino-acids, mercaptan, ammonia, carbonic acid, hydrogen, marsh gas, sulphuretted hydrogen and other evil-smelling gases. Kendall (Kendall and Farmer, 1912–13) regards all action of bacteria on nitrogenous substances as putrefaction, in distinction to their action on carbon compounds, which he terms fermentation.

*Chemical Changes in Putrefaction.**

Since the general recognition of the composition of the proteins which followed the work of E. Fischer, 1900–7, the phenomenon of putrefaction

* For a more detailed account of this subject see Barger (1914).

has been recognized as consisting in the progressive hydrolysis of the proteins followed by the decomposition of the resulting amino-acids. At the same time many non-protein constituents of the putrefying material such as the lipins, creatine and possibly the purines and pyrimidines are also attacked.

The direct connection of the products of putrefaction with their corresponding amino-acids, &c., has been experimentally determined by submitting single amino-acids to putrefaction, either by a mixed culture derived from putrefying meat or pancreas or by *B. putrificus* (itself probably a mixture of anaerobes) (for details of this work, see Barger, 1914).

Hydrolysis of proteins. It is probable that bacteria, in common with other cells, all contain proteolytic enzymes (see Hahn, 1898; Geret and Hahn, 1898) capable of producing hydrolysis of the proteins of the cytoplasm, although this has not yet been proved in every case. In addition to this the culture media in which many bacteria have been grown are found to contain powerful proteolytic enzymes. In view of the great uncertainty which exists concerning the nature, function and relationships of the proteolytic enzymes (see Oppenheimer, 1926, pp. 803 *et seq.*) only points of direct bacteriological interest are here treated (full discussions will be found in Waksman, 1922, and Oppenheimer, 1926, pp. 1129 *et seq.*).

Liquefaction of gelatin. It was early recognized that the liquefaction of gelatin (or serum-proteins) was brought about by a soluble enzyme which passed into the medium in which the organism had grown (Bitter, 1886). The change produced in the gelatin is apparently a slight degree of hydrolysis, unaccompanied by the production of ammonia or amino-acids (Kendall, Cheetham and Hamilton, 1922). Liquefaction of gelatin is used as a diagnostic test, which is usually carried out by cultivating the organism on solid nutrient gelatin at 22° C. The test may also be made with organisms which only grow slowly at 22° C. by cultivating on nutrient gelatin at 37° C. and then cooling. The liquefaction of gelatin is interfered with by the presence in the medium of sugar. In the case of *B. proteus*, this appears to be due to lessened production, or total non-production, of the proteolytic enzyme under these conditions (Kendall and Walker, 1915; Kendall, Cheetham and Hamilton, 1922; see also Berman and Rettger, 1918). Diehl (1919), confirming the earlier work of Fermi (1896), found that the liquefying enzyme in many cases (*B. pyocyaneus*, *B. prodigiosus*, *B. subtilis*) was not produced on simple inorganic synthetic media, although Braun and Cahn-Bronner (1921) obtained positive results with *B. pyocyaneus*. On a peptone medium Diehl found that these organisms both liquefied gelatin and digested caseinogen, whereas on an amino-acid medium one or other of these effects failed to appear.

Nature and effects of the proteolytic enzymes of bacteria. Even less is known of the proteolytic enzymes of bacteria than of other organisms, owing to the difficulties which arise from the small quantities of material

available. They have been studied by plating methods in the filtrates from cultures of such organisms as liquefy gelatin, in the material obtained by grinding, drying or freezing the organisms and finally by allowing the organisms to autolyse (see Oppenheimer, 1926, pp. 1129 *et seq.*). According to Dernby (Dernby and Blanc, 1921; Dernby, 1922), *B. subtilis*, *B. pyocyaneus*, *B. proteus*, *B. prodigiosus*, *B. sporogenes* and *B. histolyticus* all produce extracellular proteolytic enzymes of the same type, which liquefy gelatin and hydrolyse peptone over a wide range of hydrogen ion concentration, extending from pH 4 to 9, the optimum being a zone about pH 6 to 7. They differ, therefore, both from pepsin and animal trypsin and resemble more closely the endo-tryptase of yeast, which has its maximum effect at about pH 7, and the vegetable tryptases.

In their action on caseinogen these enzymes first produce precipitation of a curd of casein, which is then gradually dissolved (Hata, 1904), in this resembling the animal digestive enzymes. Many bacteria possess the power of digesting fibrin and denaturated egg-white as well as caseinogen and the proteins of wheat (see Crabill and Reed, 1915).

It thus appears that protein material can be rapidly hydrolysed by bacteria, yielding comparatively simple compounds (amino-acids and polypeptides) which then undergo further decomposition in the process of putrefaction.

Decomposition of amino-acids. The two chief changes involved in the decomposition of the amino-acids (and possibly the peptides) formed by hydrolysis of the proteins are decarboxylation and deamination, sometimes accompanied by reduction or by oxidation.

(1) *Decarboxylation.* (*a*) $\underset{\alpha\text{-Amino-acid}}{R\cdot CH(NH_2)\cdot COOH} = CO_2 + \underset{\text{Base}}{R\cdot CH_2\cdot NH_2}.$

(*b*) $R\cdot CH(NH_2)\cdot COOH + 2\,H = \underset{\substack{\text{Formic}\\\text{acid}}}{H_2CO_2} + \underset{\text{Base}}{R\cdot CH_2\cdot NH_2}.$

This is the origin of many bases produced in putrefaction, some of which are physiologically very active. In this way, leucine, $(CH_3)_2\cdot CH\cdot CH_2\cdot CH(NH_2)\cdot COOH$, yields *iso*-amylamine, $(CH_3)_2\cdot CH\cdot CH_2\cdot CH_2\cdot NH_2$, tyrosine yields *p*-hydroxyphenylethylamine, $OH\cdot C_6H_4\cdot CH_2\cdot CH_2\cdot NH_2$, and histidine, $C_3H_3N_2\cdot CH_2\cdot CH(NH_2)\cdot COOH$, yields histamine, $C_3H_3N_2\cdot CH_2\cdot CH_2\cdot NH_2$. Lysine, $NH_2\cdot CH_2\cdot [CH_2]_3\cdot CH(NH_2)\cdot COOH$ by a similar change may yield pentamethylenediamine or cadaverine, $NH_2\cdot CH_2\cdot [CH_2]_3\cdot CH_2\cdot NH_2$, and ornithine, $NH_2\cdot CH_2\cdot [CH_2]_2\cdot CH(NH_2)\cdot COOH$, itself derived from arginine, $NH_2\cdot C(NH)\cdot NH\cdot CH_2\cdot [CH_2]_2\cdot CH(NH_2)\cdot COOH$, by the action of the enzyme arginase, yields tetramethylenediamine or putrescine, $NH_2\cdot CH_2\cdot [CH_2]_2\cdot CH_2\cdot NH_2$. Bases of this class, which were discovered by Brieger (1885), were termed ptomaines (Selmi), and were at one time thought to be the toxic agents of food poisoning. Some of the bases have poisonous properties, but in the majority of cases of food poisoning the agent is a bacterial toxin of unknown constitution.

When the process of decarboxylation is applied to a dicarboxylic acid, a monobasic ω-amino-acid is often produced ; thus glutamic acid yields ω-aminobutyric acid (Ackerman, 1910).

$$COOH \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH \rightarrow COOH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2.$$

(2) *Deamination.* In this process simultaneous reduction occurs with the production of ammonia and an acid free from nitrogen.

$$R \cdot CH(NH_2) \cdot COOH + 2\,H = NH_3 + R \cdot CH_2 \cdot COOH.$$

This reaction is the chief source of the fatty acids so abundantly formed in putrefaction, the simple amino-acids such as glycine, alanine, valine, leucine, &c., all yielding the corresponding fatty acid with the same number of carbon atoms. Tyrosine, tryptophan, &c., undergo similar changes yielding the corresponding acids :

$$OH \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2) \cdot COOH \rightarrow OH \cdot C_6H_4 \cdot CH_2 \cdot CH_2 \cdot COOH$$

Tyrosine — *p*-Hydroxyphenylpropionic acid.

(3) *Combined decarboxylation and deamination.* A combination of both these reactions frequently occurs with the amino-dicarboxylic acids, the products being monobasic non-nitrogenous acids. Thus, aspartic acid yields propionic acid ; glutamic acid butyric acid, &c.

$$COOH \cdot CH_2 \cdot CH(NH_2) \cdot COOH \rightarrow CH_3 \cdot CH_2 \cdot COOH$$

Aspartic acid — Propionic acid

The exact reactions which lead to the production of sulphuretted hydrogen (the sulphur being derived from the cystine and in some cases the methylthionine of the protein), and the other gaseous products are not known. According to Bienstock (1899), indole was never formed in the putrefactions which he performed with *B. putrificus* (see p. 238), although it had been isolated by Salkowski and Nencki from mixed putrefactions.

An elaborate method for ascertaining the nature of the products formed by the action of bacteria on nitrogenous media has been worked out by Foreman and Smith (1928) and applied by them in investigating the action of *Staphylococcus aureus* on meat extracts.

The Bacterial Agents in Putrefaction.

Putrefaction is essentially an anaerobic phenomenon and all investigators agree in ascribing the chief chemical action to anaerobic organisms. In a mixed spontaneous putrefaction aerobes are also present (Tissier and Martelly, 1902) and probably assist in the development of the anaerobes by reducing the pressure of oxygen. The anaerobes usually found are *B. sporogenes* and allied organisms, but great variation may occur. The *B. putrificus* isolated by Bienstock (1899) and used by him and a number of other investigators for experiments on putrefaction was probably often a mixture of *B. cochlearius* or *B. tertius* with *B. sporogenes* (Medical Research Committee, 1919). Similar organisms are found in the intestine and are regarded as the active agents in intestinal putrefaction (cf. Metschnikoff, 1908).

Hydrolysis of Nitrogen Compounds.

In addition to the effects of protein hydrolysis and decarboxylation and deamination of amino-acids which are involved in putrefaction, many bacteria have the power of hydrolysing nitrogen compounds such as urea, the acid amides, nucleic acid, arginine, &c. In fact, the great majority of the enzymes found in animals and higher plants, by the aid of which changes are effected in nitrogen compounds, have also been found in bacteria.

Hydrolysis of Urea.

The hydrolysis of urea is of particular bacteriological interest. Pasteur, in 1860, recognized that the ammoniacal fermentation of urea

$$CH_4ON_2 + 2\ H_2O = (NH_4)_2CO_3$$

was due to a micro-organism, which he termed 'torule ammoniacale', and as the result of the long-continued work of Miquel (see article in Lafar, 1905) and of later workers (Tissier and Martelly, 1902; Horowitz, 1916), it is now recognized that nearly all bacteria have some power of hydrolysing urea, its degree depending to a large extent on the composition of the culture medium (see p. 246 of this work and Jacoby, 1917[1 & 2], 1918). It has also been established that the enzyme urease, to which the change is due, is, in bacteria, present in a non-extractable form (Beijerinck, 1901; Jacoby, 1917[1 & 2]), differing in this respect from the urease of plants which is readily extractable (see Lövgren, 1921, where the literature is quoted, for an account of its properties). Treatment of the organisms with chloroform, with alcohol or by drying does not seriously diminish their hydrolytic power towards urea.

Production of Ammonia.

Ammonia is a frequent product of the action of bacteria on proteins, peptones and amino-acids, both under aerobic and anaerobic conditions. The chief reactions by which it is formed are discussed under 'Putrefaction' (p. 237), and to these must be added the hydrolysis of amides: $R{\cdot}CO{\cdot}NH_2 + H_2O = R{\cdot}CO{\cdot}ONH_4$. As an example the amounts of NH_3 produced by *B. cloacæ* and *B. alkaligenes* growing aerobically in sugar-free peptone broth are quoted (Kendall and Farmer, 1912, p. 467):

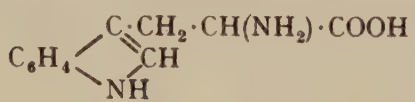
Days of incubation.	Free NH_3 as mgm. N per 100 c.cm.	
	Control 24·50. *B. cloacæ.*	Control 24·50. *B. alkaligenes.*
1	28·00	28·35
3	30·45	28·70
5	36·40	32·55
8	38·50	33·25

The production of ammonia is greatly lessened by the presence of a fermentable carbohydrate, and Kendall considers that this is due to a

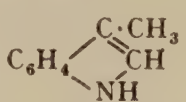
protein-sparing action of the carbohydrate (Kendall *et al.*, 1912, 1913). Raistrick, on the other hand, has shown that when pure amino-acids are used the total amount of amino-acid decomposed is increased in the presence of another source of assimilable carbon, such as glycerol; the ammonia, however, is not liberated as such but is assimilated by the organism (Raistrick, 1919, Raistrick and Clark, 1921). According to this view, therefore, carbohydrate causes an increased, and not a diminished, degree of decomposition of the amino-acid. This confirms the conclusion arrived at by Berman and Rettger (1918), and is in agreement with the later experiments of Kendall and his colleagues (1922), in which determinations of the distribution of the nitrogen of the medium show that it is the use of the nitrogenous material for the production of energy, as distinct from the synthesis of protein, which is lessened by carbohydrates. Raistrick (1919; Raistrick and Clark, 1921) has also shown that the ammonia may arise either from the amino-group of the carbon chain of the amino-acids or from the nitrogen contained in the closed ring in such compounds as histidine, $C_3H_3N_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$, and tryptophan, $C_6H_4 \cdot C_2H_2N \cdot CH_2 \cdot CH(NH_2) \cdot COOH$.

Indole Formation.

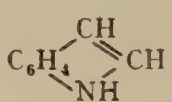
The source of the indole which is produced by many bacteria in media containing proteins or their products of hydrolysis is the amino-acid tryptophan, *l*-β-indole-α-aminopropionic acid (I):



(I.)



(II.)

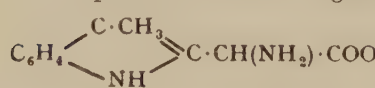


(III.)

According to the conditions of reaction this may yield by the ordinary changes characteristic of the action of bacteria on the amino-acids either skatole (methylindole) (II) or indole (III), and both these substances have been isolated from the products of protein putrefaction and occur in human faeces. A considerable number of bacteria produce indole from tryptophan, and as it is easily recognizable by qualitative tests, its formation or non-formation is largely employed as a diagnostic test. Among these are many members of the colon and para-colon, dysentery and Salmonella groups, as well as some strains of *B. proteus* and *B. ozænæ*.

The mode of decomposition of tryptophan by bacteria was clearly demonstrated by Hopkins and Cole (1903). *B. coli* when grown anaerobically on a medium containing tryptophan* produced only a

* Hopkins and Cole regarded tryptophan as skatole-aminoacetic acid,



and described the products of the action of bacteria as skatoleacetic and skatolecarbonic acid, but it was subsequently shown by Ellinger that tryptophan was indoleaminopropionic acid, the bacterial products being indolepropionic and indoleacetic acid.

trace of indole, but a large amount of indolepropionic acid, whereas when grown aerobically no indolepropionic acid was obtained but large amounts of indole and indoleacetic acid.

$$\underset{\text{Tryptophan}}{C_8H_6N\cdot CH_2\cdot CH(NH_2)\cdot COOH} \rightarrow \underset{\text{Indolepropionic acid}}{C_8H_6N\cdot CH_2\cdot CH_2\cdot COOH} \rightarrow$$

$$\underset{\text{Indoleacetic acid}}{C_8H_6N\cdot CH_2\cdot COOH} \rightarrow \underset{\text{Indole}}{C_8H_6N\cdot H}$$

All these substances had previously been isolated from the products of putrefaction of proteins by Salkowski (1885), Nencki, and Brieger.

It is interesting to note that this mode of decomposition differs from that produced by yeast, which results in the formation of tryptophol, $C_8H_6N\cdot CH_2\cdot CH_2\cdot OH$ (F. Ehrlich, 1912).

The Salkowski test for indole consists in the production of a red ring when one-third of its volume of a mixture of equal volumes of sulphuric acid and water is added to the culture, and a 0·02 per cent. solution of potassium nitrite is then run on to the surface of the mixture. These reagents, however, also yield a coloration with indoleacetic acid. On the other hand, Ehrlich's test for indole in urine (1901), which was applied to bacterial cultures by Böhme (1901), is strictly specific, gives a negative result with indoleacetic acid and can be used quantitatively (Herter and Foster, 1905–6). This test consists in the production of a red coloration when an alcoholic solution of the hydrochloride of *p*-dimethylamino-benzaldehyde, with or without a saturated solution of potassium persulphate as oxidizing agent, is added to the culture.

Production of Indoleacetic Acid.

Bacteria which produce this substance but not indole yield cultures in peptone water, or in a suitable medium to which tryptophan has been added (Frieber, 1921), which give the nitrite reaction, but not Ehrlich's reaction.

The fact that a bacillus produced a substance which gave the nitrite reaction but was not indole seems first to have been observed by Hewlett (1900, 1901) with *C. diphtheriæ*. Steensma (1906) noted the same phenomenon with a strain of *B. proteus*, and other observers subsequently confirmed this. Hewlett isolated the substance responsible for the reaction, and found it to be the compound now known as indoleacetic acid, whilst A. Berthelot (1913) isolated this same substance from cultures of *B. proteus*.

According to Neisser (1921) and Frieber (1921) nearly all non-indole-producing organisms are able to carry the decomposition of tryptophan as far as the indoleacetic acid stage, at the same time assimilating the nitrogen of the amino-group which is liberated, but are unable to convert this substance into indole.

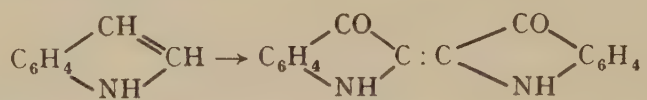
The reason why so many pathogenic bacteria, which are now known to produce indoleacetic acid, were generally regarded as negative to the nitrite test is that the formation of indoleacetic acid in the usual media,

which are poor in tryptophan, is often very slow. Old cultures of many of these were, however, found to give the nitrite test (see Marshall, 1907). The formation of indole is inhibited by the presence of glucose, or any sugar fermentable by the organism (Frieber), but under these circumstances the usual formation of indoleacetic acid occurs, only the further stage by which this substance passes into indole being inhibited. According to Neisser and Frieber this is due to the fact that the glucose provides more readily assimilable carbon than does the indoleacetic acid ; as soon as all the glucose has been decomposed, indole is produced as usual.

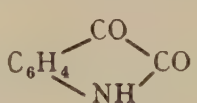
Cholera-red reaction (Nitrosoindole reaction). Some bacteria, especially those of the *Spirillum* class, reduce the nitrate of the medium to nitrite (Petri, 1890), and at the same time produce indole. When the medium is subsequently acidified the red coloration characteristic of Salkowski's test for indole is produced. This reaction is used as test for the presence of the cholera vibrio (for optimum conditions, see Groenewege, 1920 ; Bleisch, 1893), but is by no means specific, and also occurs with other species of *Vibrio*, in old cultures of *C. diphtheriæ* (Palmirski and Orlowski, 1895), and with some bacteria of hæmorrhagic septicæmia (Voges and Proskauer, 1898).

Oxidation and Destruction of Indole.

Certain bacteria can oxidize indole with formation of colourless compounds (see Gray, 1928) whilst two species have been isolated which convert it into indigotin.



This oxidation can only be effected in solutions containing 0·01 per cent. of indole when some other source of carbon (such as glycerol) is available for the organism ; on the other hand, no further source of nitrogen is required. The oxidation does not proceed further and no isatin



is produced.

Production of Phenol.

Phenol occurs in the intestinal contents of normal man and, in the form of phenolglycuronic acid, in normal urine. Its origin has been traced (see Rhein, 1917, where the literature is quoted) to bacterial action in the intestine. Many organisms of the coli group are phenol producers. Among them are *B. coli phenologenes* (Rhein, 1917, 1918 ; Berthelot, 1917) and *B. enteritidis phenologenes* (Sieke, 1921), which are allied to *B. coli communis* and *B. enteritidis* (*B. paracoli*). *B. coli phenologenes* does not produce indole, but certain strains of intestinal *B. coli* produce both phenol

and indole (Salus, 1922). The source of the phenol is the tyrosine of proteins, *p*-hydroxybenzoic acid being probably an intermediate product.

$$OH \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2) \cdot COOH \rightarrow OH \cdot C_6H_4 \cdot COOH \rightarrow OH \cdot C_6H_5.$$

The culture is tested for phenol by adding a few drops of 10 per cent. formaldehyde and pouring it on to concentrated sulphuric acid ; if phenol be present a violet-red ring is produced with a whitish opalescence above it. In presence of indole, which gives a somewhat similar coloration with formaldehyde, the more specific reaction of Berthelot-Lex is to be preferred. To 10 c.cm. of a culture cooled to 10° C. are added 0·1 c.cm. of 10 per cent. NaOH and 0·5 c.cm. of a freshly prepared 0·5 per cent. solution of *p*-aminophenol, and a few drops of NaClO solution are then run to the bottom of the solution. If phenol be present a dark blue ring is formed (due to the formation of indophenole). Both this test and the formaldehyde test are better done in the distillate from the culture.

These bacteria occur very widely in the intestine (Neisser, 1921), both of normal and pathological cases, and the property of phenol formation is quite as characteristic as that of indole formation. Some bacteria produce both indole and phenol (Sieke, 1921), this having been found to be the case with some strains of fowl cholera, and of *B. ozænæ*.

Decomposition of Phenol and Other Aromatic Compounds.

An organism capable of decomposing phenol was isolated from sewage by Fowler, Ardern and Lockett (1911). The fact that in soil many aromatic compounds, some of them powerful antiseptics, undergo oxidation has been familiar for some time, and it has now been shown (Gray and Thornton, 1928, where the literature is quoted), that bacteria capable of decomposing phenol, *m*-cresol and naphthalene are very widely distributed, particularly in arable land. No less than 25 species, falling into 7 genera, capable of this kind of action, have been isolated, some of which can oxidize *o*- and *p*-cresol, resorcinol (*m*-dihydroxybenzene) and phloroglucinol (1 : 3 : 5-trihydroxybenzene) and even toluene. The organisms can grow in a synthetic medium with no other source of carbon than is supplied by these aromatic compounds. A concentration of 0·1 per cent. of phenol could be utilized by some of them, whereas 0·2 per cent. proved fatal.

Production of Enzymes by Bacteria.

Bacteria, like other living organisms, carry out a large proportion, if not all, of the chemical changes which occur in the metabolism and growth of the cell by the aid of enzymes. This aspect of the subject has received attention in the foregoing pages. The detection of such bacterial enzymes as pass into the culture medium (exo-enzymes or soluble enzymes) is effected by the usual methods of enzyme chemistry. The demonstration of the insoluble or endo-enzymes is in some cases difficult owing to the small size of the organism, and hence the endo-enzymes of bacteria are less

well known than those of some other micro-organisms, such as yeast and the moulds (e.g. *Mucor, Aspergillus, Penicillium,* &c.).

Enzymes can often be demonstrated in bacteria by the use of plating methods, which were introduced for this purpose by Eijkman (1901, 1903[1 & 2], 1903) and have since been employed by many workers (see Crabill and Reed, 1915; Allen, 1918; Kellerman, *et al.*, 1913). A comprehensive review of the whole subject of enzymes in micro-organisms is given by Waksman (1922, 1928).

The influence of the composition of the culture medium on the production of enzymes by bacteria. Abundant evidence exists, expecially for the moulds, that the composition of the culture medium has a great effect both on the nature and the quantity of the resulting enzymes. Although many enzymes are found in bacteria growing in simple synthetic media (Braun and Cahn-Bronner, 1921), it appears to be well established that the presence of an enzyme substrate in the medium stimulates the production of the corresponding enzyme (see Waksman, 1922, pp. 274–5), but the experimental work on this subject as regards bacteria is often confused by failure to allow for increased growth of the organism (see e.g. Glaessner and Roscules, 1906).

A more fundamental effect has also been observed in which some special substance seems to be essential for the production of a certain enzyme. *B. proteus* (Jacoby, 1917, 1918) produces catalase abundantly on Uschinsky's synthetic medium and still more abundantly if ammonium lactate be added, whereas on these media only traces of urease are formed. If, however, *l*-leucine or *d-iso*leucine be added a vigorous production of urease results. Other amino-acids, such as glycine, alanine, tyrosine, &c., are ineffective, so that the observation seems to point to some special structural relation between the enzyme and the leucine.

The production of the proteolytic enzymes has already been discussed on p. 238.

Production and Decomposition of Stereoisomerides by Bacteria.

A special case of bacterial metabolism which is of great biological interest is the selective decomposition or production of stereoisomerides. The theory of the action is comparatively simple. It is beyond doubt that the enzymes which catalyse the reactions of reduction, oxidation, fermentation and synthesis, by which substances are decomposed and new compounds formed in the living cell, are optically active and have an asymmetric structure. Hence, when the organism acts on a mixture of optically opposite stereoisomerides the enzyme may combine with the two isomerides at different rates, forming two distinct compounds, which are stereochemically not mirror images, and which may, therefore, have different degrees of stability and decompose at different rates.

Supposing, for example, that the enzyme (E) is lævo-rotatory (E—), the two compounds formed with the two stereoisomerides S— and S+ will

be respectively represented as E— S— and E— S+. In general, the two antipodal optically active stereosiomerides will be decomposed at different rates. Any degree of difference in rate may actually occur and in extreme cases it may be very small, so that very little selection is observed, or very great, so that only one constituent disappears, and the other remains virtually intact. The action is closely analogous to the asymmetric action of enzymes which was first demonstrated *in vitro* by Dakin (1903) in the case of lipase acting on esters of mandelic acid, and has since been frequently observed.

The production of an optically active substance from an inactive substrate may be explained on the same principle, one of the two stereoisomerides being predominantly formed.

Asymmetric Decomposition of Stereoisomerides.

Many striking examples of this action are afforded by yeasts and moulds. Thus the optical antipodes of the naturally occurring hexoses, *l*-glucose, *l*-fructose and *l*-mannose are not fermented by yeast, whereas the *d*-forms which occur in plants and animals are readily fermented (Fischer, 1890). The same considerations apply to the action of yeasts on the amino-acids, the naturally occurring isomerides such as *l*-leucine, *l*-tyrosine, &c., being decomposed in the manner already described (p. 214), whilst the antipodal forms are not attacked. This selective action can, in fact, be used to prepare the more resistant isomerides from the *dl*-mixture of amino-acids obtained by synthesis (F. Ehrlich, 1906). Moulds generally act on both isomerides, but at different rates, a mixture of unequal quantities of the two stereoisomerides resulting (see McKenzie and Harden, 1903). The classical instance of their action is afforded by the resolution of racemic acid by Pasteur (1860) by the use of *Penicillium glaucum*.

The selective action of bacteria has also frequently been observed. One of the best established instances is the asymmetric decomposition of glyceric acid by *B. ethaceticus*, an organism which attacks the *l*-constituent the more rapidly and may be employed for the preparation of *d*-glyceric acid (Frankland and Frew, 1891).

A few other actions of the same sort may be quoted. *d*-Tyrosine may be prepared by the action of *B. proteus vulgaris* on *dl*-tyrosine, a yield of 65 to 70 per cent. of the theoretical being obtained (Tsudji, 1917), and this same organism also acts asymmetrically on *dl*-phenylalanine (Amatsu and Tsudji, 1918) and on *dl*-naphthylalanine (Sasaki and Kinose, 1921), the *d*-isomeride being less easily attacked in both cases. The asymmetric hydrolysis of the synthetic optically inactive polypeptide *dl*-leucylglycine is effected by killed *B. coli communis* and *S. aureus*, *l*-leucine being preferentially split off (Mito, 1917). Mandelic acid (Lewkowitsch, 1882) and propylene glycol (Le Bel, 1881) are both decomposed asymmetrically by *B. termo*, and these compounds, in addition to racemic acid and inactive lactic acid, have been asymmetrically

decomposed by various unidentified bacteria (Le Bel, 1893; Frankland and Macgregor, 1893; Lewkowitsch, 1883). Inactive butylene glycol is selectively oxidized by the sorbose bacillus or by *Mycoderma aceti*, the *l*-form being converted into acetoin, $CH_3 \cdot CO \cdot CH(OH) \cdot CH_3$, whilst the *d*-form is unattacked (Kling, 1905, 1906).

Production of Optically Active Substances.

The production of optically active substances by bacteria is explained on the same principle as the selective decomposition of stereoisomerides. The case of lactic acid has attracted much attention, owing to the frequency of the occurrence of this substance as a product of bacterial action. It is now thought (p. 220) that this acid is produced from an optically inactive precursor, methylglyoxal, by intramolecular oxido-

$$CH_3 \cdot CO \cdot CHO + H_2O = CH_3 \cdot CH(OH) \cdot COOH$$

reduction, the central carbon atom being rendered asymmetric. As the change is enzymic, the conditions exist for the production of either of the two optically opposite forms or of a mixture of the two in any proportions. Actually the optical character of the lactic acid formed by bacterial action varies very greatly, not only with different organisms, but with the same organism under different conditions (Péré, 1892, 1896, 1898; Harden, 1901). It has occasionally been used to characterize certain organisms, especially in the vibrio and typhoid-coli groups (see Gosio, 1894; Rontaler, 1895). Optically inactive *dl*-lactic acid, a mixture of the optically active acids in equal proportions, is produced by *B. butyricus*, and the commercial 'fermentation' lactic acid is also usually inactive. The product obtained by Neuberg (Neuberg and Simon, 1927; Neuberg and Gorr, 1925[1 & 2],) by the direct action of bacteria on methylgloxal was also inactive. The *d*-acid, which, under the name sarco-lactic acid, is well known as the form which is found in muscle, is produced, sometimes alone, sometimes along with a smaller amount of the *l*-acid, by the spontaneous souring of milk (see Kozai, 1899; Günther and Thierfelder, 1900; Utz, 1904; Thiele, 1904). The *l*-acid is produced by many organisms of the vibrio and typhoid-coli groups and by *B. pestis* (Gosio and Bignelli, 1898).

Hydrogen-Ion Concentration and Bacterial Metabolism.

For all bacteria there exist limits of hydrogen-ion concentration [H˙], between which alone life, with its accompanying phenomena of growth and metabolism, is possible. Moreover, in most cases, as the result of the metabolic activity of the organism the [H˙] of the culture medium undergoes variations which may be due to a number of different causes, e.g. production or removal of acids, or alkaline substances, or decomposition of salts of organic acids. These changes may be progressive and

go so far that the life processes of the organism are checked, or they may only proceed to a certain limit within the range unharmful to life. Finally, in some cases the nature of the metabolic processes and of the resulting products may be profoundly modified by the [H˙] of the medium.

Changes of [H˙] of the Medium produced by Bacteria.

Increase of [H˙]. Production of Acid.

A striking feature of the metabolism of many micro-organisms, especially in media containing as substrate sugar or other fermentable compounds, is the production of acid. This may be formed either by the fermentative decomposition of the substrate, as in the case of *B. coli*, or by its oxidation, as with *B. aceti*. The usual course of such a change is that as the [H˙] rises the rate of production of acid becomes slower, until finally no further change occurs.

The absolute amount of acid required to attain this point depends on the degree of buffering afforded by the medium. Thus, whereas 1 per cent. glucose-peptone acquired a pH of 5 when lactic acid was added up to a concentration of 0·007 *N*, the same medium with an addition of Na_2HPO_4 required a concentration of 0·06 *N* lactic acid to bring it to the same pH (Clark, 1915). The substances chiefly responsible for the ' buffering ' of meat extract over the region in which bacteria are active seem to be the phosphates (pH 7·2 to 6·3) and creatinine (pH 5 to 4·5) (Foreman and Smith, 1928). Peptone also has a ' buffering ' effect, which is of importance in peptone-broth, especially in the region pH 7·8 to 8·25 (Bunker, 1919).

The factors which determine the value of this final [H˙] have in several instances been carefully investigated. It was at first claimed that this value was a physiological constant for the organism (Michaelis and Marcora, 1912; Clark, 1915), and that the rate at which it was attained could be used for diagnostic purposes, for example, in the differentiation of various closely allied members of the *B. typhosus* group (Cole and Onslow, 1916). It has, however, been found that both the final [H˙] and the rate at which it is attained depend upon a number of different factors, and, although constant for a given medium, are by no means physiological constants (Wolf and Harris, 1917; Wyeth, 1918). The chief factors which determine the final [H˙] are the following: (1) The initial [H˙] of the medium; (2) The extent to which the medium is buffered by the presence of salts of weak acids; (3) The nature of the acid used to produce the initial [H˙] of the medium.

Table V, taken from the results of Wyeth (1918) illustrates all these points in the case of *B. coli*. The ' critical pH ' is the pH for any particular medium and acid beyond which the slightest increase of acidity completely inhibits growth. It is an average number derived graphically from a series of experiments.

TABLE V.

Medium.	Acid used to produce initial pH.	Initial pH.	Final pH.	Critical pH.
2 per cent. glucose-peptone.	HCl	—	—	4·27
	Lactic	—	—	4·47
	Acetic	—	—	4·68
	,,	7·14	4·53	—
	,,	5·39	4·60	—
	,,	4·83	4·77	—
2 per cent. glucose-phthalate.	HCl	6·95	5·73	—
	,,	6·26	4·99	—
	,,	5·06	4·74	—
	,,	4·91	4·71	—
	,,	—	—	4·55
	Lactic	—	—	4·73
	Acetic	—	—	4·83

The initial addition of acid to a medium causes a definite latent period of growth, which becomes longer as the addition of acid becomes greater. The final pH, in the case of *B. coli*, was found to be the same for all strains of the organism when tested in media of the same composition.

Decrease of [$H^{\cdot}$].

Many bacteria render the medium more alkaline, this being the case, for example, in litmus-whey with *S. pyogenes aureus*, many Streptococci, *B. pyocyaneus*, *B. proteus*, &c. (Petruschky, 1889) and for a large number of organisms in peptone broth (Kendall *et al.*, 1912, 1913). In other cases the medium is first rendered more acid, but a reversal then occurs and the reaction becomes gradually less acid and finally alkaline. A well-known instance is afforded by *C. diphtheriæ* growing in peptone broth, and the same phenomenon is shown by *B. paratyphosus B*, and other organisms in litmus milk.

Production of ammonia or bases. The alkaline reaction produced by many bacteria is due to the formation of ammonia or amines from the nitrogenous matter of the medium. The chemical changes which result in the production of these alkaline substances of culture media have already been discussed (pp. 239, 240). Under aerobic conditions the resulting carbon compound may be oxidized and thus converted into carbon dioxide and water. In general, it may be said that the products of such reactions are, on the one hand, ammonia or an organic base, and on the other, organic residues of various constitution or carbon dioxide. The effect of the change on the pH will obviously depend on the relative acidities of the products, on the nature of the substance decomposed and on the medium. Thus, to take a simple example,

the conversion of glycine by decarboxylation into methylamine $NH_2 \cdot CH_2 \cdot COOH \rightarrow NH_2 \cdot CH_3 + CO_2$ would cause a rise of pH, a solution of glycine being about neutral and one of methylamine bicarbonate alkaline.

Oxidation of salts of organic acids. A second source of alkalinity or diminished acidity is the conversion of acids into less acid products by fermentation, decomposition or oxidation. This will produce a rise in pH whether the acid be free or present as a salt (Ayers and Rupp, 1918). Thus, formic acid and formates are decomposed by *B. coli* into hydrogen and carbonic acid or a carbonate,

$$H \cdot COOH = H_2 + CO_2$$
$$H \cdot COONa + H_2O = H_2 + NaHCO_3$$

a rise in pH in each case following the conversion of the comparatively strong formic acid into carbonic acid.

An interesting instance of this change is afforded by the production of alkalinity in milk by many bacteria without production of ammonia or peptization of caseinogen, which is due to the oxidation of the citrate of the milk with formation of the less acid carbonate (Ayers, Rupp and Johnson, 1919).

Reversal of reaction. The foregoing phenomenon takes a large share in the reversal of reaction which occurs in some bacterial cultures, such as those of *C. diphtheriæ* and of *Micrococcus aureus* (Kendall, Farmer, Bagg and Day, 1912). Acids are first produced from some fermentable constituent of the medium and a rise in $[H^+]$ results. These acids are subsequently decomposed and at the same time other constituents of the medium are converted into less acid products, among which is usually a considerable amount of CO_2, and ammonia is also frequently formed, the result being that the $[H^+]$ gradually falls. The following example (Table VI), quoted from Wolf's observations on *C. diphtheriæ* (1922) illustrates the point.

TABLE VI.

Hours.	pH.	CO_2 c.cm. per 100 c.cm.	Volatile acids c.cm. N/10.	Fixed acids per 100 c.cm.
Control ..	7·8	3·0	3·6	27·2
24 ..	7·5	—	4·4	20·5
48 ..	7·3	—	14·8	6·6
96 ..	7·5	—	15·8	7·4
120 ..	8·1	18·0	7·2	4·5
192 ..	8·5	27	7·2	2·9

Only a small amount of ammonia, of the order of 3 to 5 mgm. per 100 c.cm., was produced in these experiments, but amounts up to 31 mgm. per 100 c.cm. have been recorded by Abt and Loiseau (1925). A similar phenomenon occurs with the organisms of the *typhosus-paratyphosus*

group and has been used for their differentiation (Cole and Onslow, 1916). In 0·2 per cent. glucose-tryptic broth *B. paratyphosus B* produces an alkaline reaction to phenol red after 24 hours at 37°, whereas similar cultures of both *B. typhosus* and *B. paratyphosus A* remain acid. In 0·2 per cent. dulcitol broth, on the other hand, *B. typhosus* becomes alkaline under these conditions, whereas *B. paratyphosus A* remains acid.

An application of the same principle has been suggested for the differentiation of organisms of the type of *B. lactis aerogenes* from those of the type of *B. coli* (Clark and Lubs, 1915, 1917). In 0·5 per cent. glucose, 0·5 per cent. Witte peptone and 0·5 per cent. K_2HPO_4 organisms of the former group ferment the whole of the dextrose with the production of a small amount of acid and then produce a reversal, so that at the end of 3 to 5 days at 30° C. the culture is found to be alkaline to *p*-nitrophenol (pH about 6 to 7), whereas coli-like organisms in this medium produce a permanent acidity to this indicator (pH about 4·7).

Effect of [H·] on Metabolism.

Apart from the general effect of a high concentration of hydrogen or hydroxyl ions, which diminishes the metabolic rate and puts an end finally to the metabolism and the life of the organism, the hydrogen ion concentration in some cases has a profound effect on the nature of the metabolic products. An instance of this, which is of great practical importance and has been carefully investigated, is the production of toxin by such organisms as *C. diphtheriæ* and *B. tetani* (q.v. Vol. V and Vol. III). In each of these cases it has been found that toxin formation is favoured by a particular range of hydrogen-ion concentration and ceases or is greatly diminished beyond these. *C. diphtheriæ* produces no toxin in media with pH below 7, and gives its best yield in a medium of initial pH 7·2 to 7·5 which gradually changes to 8·8·3 in the course of 8 days (Bunker, 1919; Dernby and David, 1921), whereas the optimum for toxin production by *B. tetani* is about pH 7 (Dernby and Allander, 1921), and the pH must not be allowed to fall below 6·8.

Another instance of a different character is afforded by the acetone-butyl alcohol fermentation produced by *B. amylobacter* (see p. 229). When the reaction is kept approximately neutral by the addition of chalk, the products, in addition to CO_2 and H_2 which are evolved, consist largely of acetic and butyric acids, whereas in the absence of chalk the pH first falls and then rises to a constant value, and only small quantities of these acids are found in the final dissolved products, which consist almost exclusively of acetone and butyl alcohol (Reilly *et al.*, 1920).

Accessory Growth Factors.

The higher forms of animal life require for their proper development and maintenance, in addition to foodstuffs capable of supplying the necessary amounts of N, C, and other elements, and of yielding a sufficient quantity of energy by their oxidation, minute quantities of materials

known as vitamins or accessory food factors. They are unable to produce these and are dependent for them on the vegetable kingdom or on the direct action of light. Whether the higher plants also require such adjuvant substances, and if so, whence they derive them, are disputed questions. It has, however, been demonstrated that certain species of yeast, particularly those used in brewing and distilling, generally termed culture yeasts, in contradistinction to the wild yeasts which occur on fruits and in the soil, &c., either cannot be grown at all or only with great difficulty in many types of synthetic media composed of pure sugars and inorganic salts, in which nitrogen is supplied either as an ammonium salt or some other chemically pure compound (Wildiers, 1901 ; see Tanner, 1925, for an account of this subject, and literature up to 1924).

Although several investigators claim to have isolated substances having the properties assigned to Wildiers' Bios, its chemical structure is still unknown, and it is still uncertain whether or not it may be identified with one of the vitamins recognized as necessary for the mammalian organism, although the trend of evidence is against such an identification.

With regard to bacteria, the existence of such accessory growth factors has been much discussed, since Jordan Lloyd, in 1916 (1916), showed that the *Meningococcus* required for its cultivation outside the body a medium which contained a constituent not present in ordinary peptone broth agar but present in blood and some other natural materials. (For a comprehensive account of this subject see Knorr, 1925, with literature up to 1924).

As the result of much investigation it has been found that some bacteria can be cultivated continuously on synthetic media composed entirely of substances of known composition and presumably free from any growth-promoting factor. For example, the media used by Hosoya and Kuroya in their exhaustive researches on this subject (1923) were Uschinsky's and Fraenkel's of the following composition (Table VII).

TABLE VII.

	Uschinsky.	Fraenkel.
Glycerol	35 gm.	—
Magnesium sulphate	0·3 gm.	—
Calcium chloride	0·1 gm.	—
Sodium chloride	6·0 gm.	5·0 gm.
Ammonium lactate	6·5 gm.	6·0 gm.
Dipotassium hydrogen phosphate ..	3·0 gm.	2·0 gm.
Asparagine	4·0 gm.	4·0 gm.
Distilled water	1,000 c.cm.	1,000 c.cm.
pH adjusted to	6·8 to 7·0	6·8 to 7·0

These were used as basal solutions to which 0·5 per cent. glucose was added.

When a sugar is used as a constituent of the medium it is essential that it be first freed from traces of a growth-promoting substance which appears

to be invariably present. This is done in the case of cane-sugar by repeated treatment with fuller's earth, followed by recrystallization from alcohol (Funk and Freedman, 1923); glucose may be recrystallized successively from 80, 75, 70 and 65 per cent. alcohol (Reader, 1927).

In order to eliminate the effect of carrying over a portion of the active material in the inoculum, the system of continuously repeated subculture was employed by Hosoya and Kuroya (1923), and by Robertson (1924), as many as 50 to 70 successive subcultivations being used.

On such nutrient media as these many species of bacteria have been found to be susceptible of continual subcultivation. *B. coli communis*, *Vibrio choleræ*, *B. pyocyaneus* and *B. anthracis* (Hosoya and Kuroya), *B. prodigiosus*, *B. proteus*, *B. subtilis*, *Sarcina lutea* (Robertson); while a number of others, *B. typhosus*, *B. paratyphosus A*, *Meningococcus*, *C. diphtheriæ*, *B. tetani*, *B. œdematis maligni*, and *B. welchii*, *Staph. pyogenes albus* and *citreus*, *B. dysenteriæ* (Shiga) grew in the above media with the addition of certain pure amino-acids (Hosoya and Kuroya, 1923). On the other hand *Streptococcus hæmolyticus* and *Pneumococcus* have not been observed to grow on any medium containing only substances of known composition. The same is also true for the hæmophilic bacteria.

The organisms which are capable of continued cultivation on synthetic media are nevertheless greatly influenced by the presence in the medium of extracts of many animal and vegetable tissues, inasmuch as their growth is greatly accelerated in the early stages, although the final result is not affected (Hosoya and Kuroya). The hæmolytic streptococci and pneumococci, in contrast to the organisms just referred to, do not grow on synthetic media and appear to be absolutely dependent on the presence of a growth-promoting factor. This can be supplied from many sources. Thus, according to Hosoya and Kuroya (1923), these bacteria grow in ordinary routine broth, which contains the active principle, whereas no growth occurs when the broth is autoclaved at pH 7 at 140° for 2 hours. The addition of Tsukiye's 'Vitamin B'* (1922), or of a solution of this material autoclaved at 140° C. for 2 hours (pH 7), to this autoclaved broth rendered the medium capable of supporting the growth both of hæmolytic streptococci and of pneumococcus.

Properties of the Substance promoting the Growth of Hæmolytic Streptococci and Pneumococcus.

The close dependence of these organisms on the presence of a growth-promoting factor has enabled Hosoya and Kuroya to determine the general properties of this factor with some degree of precision as well as to

* This is a preparation made by extracting rice bran with three parts of warm water, precipitating successively with alcohol, basic lead acetate and phospho-tungstic acid and decomposing the final precipitate with baryta. From the filtrate, after treatment with silver nitrate and nitric acid, ammoniacal silver nitrate solution precipitates a silver compound, which, when decomposed by H_2S yields a solution of the 'vitamin B', which is finally precipitated by the addition of 10 volumes of alcohol (Tsukiye, 1922).

differentiate it from the bios of Wildiers, which promotes the growth of yeast, and from the antineuritic factor (vitamin B_1) of yeast, all three being present in the preparation termed Tsukiye's vitamin. The properties of these three factors, as determined by Hosoya and Kuroya, are summarized in Table VIII.

TABLE VIII.

	Factor for hæmolytic streptococci and pneumococcus.	Bios.	Vitamin B_1.
Effect of temperature.	Stable at pH 7 at 160 to 162° C. for 2 hours, destroyed at 185° C. in 2 hours.	Stable at pH 7 at 185° C. for 2 hours.	Destroyed at pH 7 in 2 hours at 140° C.
Effect of alkalis..	Rapidly destroyed by dilute alkalis at boiling point.	Stable for 2 hours at 140° C. in *N* alkali solution.	
Adsorption ..	Adsorbed by fuller's earth and by animal charcoal at pH 7.	Adsorbed by animal charcoal but not by fullers' earth at pH 7.	

This factor is probably identical, or closely allied, to that which stimulates the early growth of such organisms as *B. coli communis*, *B. pyocyaneus*, *B. typhosus*, &c., and has been found by various investigators to be present in ordinary beef broth culture media and in many animal and vegetable extracts, fruit juices, &c. (Weichardt, 1920 ; Davidsohn, 1924 ; Leichtentritt and Zielaskowski, 1922 ; Reader, 1928).

The question of the identity of this factor as derived from the different sources and the further question of its relation to the vitamins necessary for the growth and maintenance of mammals have been much discussed. The bacterial growth-promoting factor appears to have no relation to vitamins A, D and C, although it accompanies the last-named in many vegetable extracts, fruit juices, &c. It is also associated with the water-soluble vitamin B of McCollum (now known to be complex and to contain at least two constituents, vitamins B_1 and B_2 of the English School, vitamins F and G of Sherman) and with the yeast-growth-promoting factor (Wildiers' Bios, Funk's vitamin D) in many sources, but there seems little doubt that these principles (as found by Hosoya and Kuroya) are distinct and differ especially in their stability to heat and alkalis, and in their behaviour to adsorbents, as well as in their biological effect.

The question of the relation of the factor which promotes the growth of *Streptothrix corallinus* (Reader, 1928) to the antineuritic vitamin B_1 has been carefully examined by Peters and his co-workers (Peters, Kinnersley, Orr-Ewing and Reader, 1928). In the process of concentrating vitamin B_1 (the antineuritic factor) from an extract of yeast by adsorption on norite charcoal after removal of all material precipitable by lead acetate, baryta and mercuric sulphate, this principle is obtained free from the more thermo-stable vitamin B_2 (Chick and Roscoe, 1927). It still, however, contains the substance which promotes the growth of *S. corallinus* and this accompanies it during the further processes by which both principles become concentrated about 100 times. Treatment of these relatively pure fractions with alkali, however, inactivates the vitamin B_1 (i.e. renders it incapable of curing polyneuritic pigeons), but leaves the growth-producing property for *S. corallinus* unaffected. The close association of these two factors is consistent with the idea (see Peters *et al.*, 1928) that the same nucleus may be involved in each case, some slight change making the factor unavailable for the pigeon though not for the micro-organism. Definite proof of this is, however, as yet wanting.

Production of the Growth-promoting Factor for Bacteria by Micro-organisms.

The interesting question whether bacteria capable of continuous sub-cultivation on synthetic media are independent of any growth-promoting substance (although their growth is accelerated by it) or whether they synthesize it for themselves has also been studied by Hosoya and Kuroya (1923). They grew *B. coli communis* on a synthetic medium through 70 subcultivations and finally on an agar medium made from the same nutrient solution. The bacterial film was then emulsified in water, allowed to autolyse at 37° C. for a week in presence of toluene, made to 70 per cent. with alcohol and boiled at 85 to 90°C. for 4 hours. The alcohol was then removed by evaporation, leaving about 200 c.cm. extract for every 1·8 gm. of dried bacteria taken. This extract had a powerful effect on the growth of hæmolytic streptococci, *C. diphtheriæ*, *Pneumococcus*, *B. coli* itself and similar organisms, as well as on yeast. It, therefore, presumably contained the growth-promoting factor for bacteria and for yeast. It also promoted the growth of rats on a diet free from vitamin B.

Similar results with reference to the growth of yeast have been observed with extracts of many other bacteria, and some moulds (see Robertson, 1924; Goy, 1921, 1922).

Hæmophilic Bacteria.

It appears from the foregoing that all species of bacteria which will grow on peptone-broth media are also capable of growth either in an appropriate synthetic medium or in a synthetic medium to which a growth-promoting extract has been added.

In the case of the hæmophilic bacteria (*B. influenzæ*) Pfeiffer; *B. hæmoglobinophilus canis*), the conditions required for growth are more

complex. The details of the method of investigation and of the results obtained are to be found in Volume II, but the position may be briefly summarized here.

B. influenzæ requires for its growth, in addition to the constituents of a synthetic medium, two factors (Fildes, 1921 ; Thjötta and Avery, 1921). One of these, usually termed the V (vitamin) factor is present in many vegetable extracts and is produced by many bacteria. The production of this V factor by bacteria gives rise to the ' satellite ' growth which has been largely used in the investigation of hæmophilic bacteria. On a medium such as blood-agar, containing only a trace of blood, these organisms will not grow, owing to the absence of a sufficient concentration of the V factor. If, however, a culture of another organism which produces the V factor, such as *Staph. pyogenes aureus* be made on the medium, the hæmophilic organisms then grow as ' satellites ' in isolated colonies at some distance from the colonies of the second organism (Riesenwachstum (giant growth) of Grassberger, 1897). This factor also occurs in many vegetable extracts and is thermo-labile, being inactivated in 15 minutes at 90° C., very sensitive to alkali, less so to acid, diffusible through parchment and not readily susceptible to atmospheric oxidation. It, therefore, differs from all the vitamins and growth-promoting factors hitherto described, although it has been thought, on inadequate grounds, by some workers to resemble in some respects vitamin C (Knorr, 1924 ; Kollath, 1924, 1925, 1926).

The second factor, X, occurs in blood, but is also present in potato (Thjötta and Avery, 1921). As potato also contains the V factor, sterile potato provides a medium on which *B. influenzæ* grows readily. The X factor is also produced by some bacteria (probably by their action on iron compounds present in the medium) and such bacteria also give rise to a satellite phenomenon (Neisser's Ammenwachstum, foster-growth) in which the influenza bacilli only grow in admixture with, or in immediate proximity to, the other organism. In blood it is thermo-stable. It is a substance containing iron, and probably acts as a catalyst by activation of oxygen, and media containing it show the benzidine test for active oxygen (Olsen, 1920 ; Fildes, 1921–22). According to Kollath (1926) the X factor can be replaced in satellite experiments by potassium ferrocyanide which has been irradiated with ultra-violet light or by a mixture of non-irradiated potassium ferrocyanide with irradiated lecithin or with acrolein (which Kollath regards as one of the products formed by irradiation of lecithin).

His view of the mode of action of the accessory factors in aiding the growth of the influenza bacillus, which is largely speculative and which he applies to the general question of the nature of vitamins, is that an aldehyde is split off from a phosphatide by the action of ultra-violet light. This activates towards oxygen a mineral salt (of iron or manganese) and the latter by combining with a water-soluble phosphatide is enabled to enter the cell, where it transfers its oxygen to the plasma.

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CHAPTER VII. BACTERIAL RESPIRATION.

By J. W. McLeod (University of Leeds).

All studies of animal and vegetable respiration involve the consideration of two main problems: that of the mechanism of gaseous exchange between the tissues and the surrounding atmosphere, and the much more complex one of intimate cellular respiration. In the latter problem is involved the whole scheme of chemical reactions by means of which the cell is supplied with the energy it requires. It is this problem of intimate cellular respiration which chiefly concerns us in the case of bacteria, and, owing to their existence in the shape of isolated cells and to the ease with which these cells are manipulated in culture, they afford a specially suitable material for this study.

At the very beginning of the exact study of bacteria it was shown by Pasteur that one of the conceptions of biology then considered fundamental—no life without free oxygen—had to be abandoned; and it is probable that an extended and intensive study of bacteria will further illuminate ideas on cellular respiration derived from the study of plants and animals.

Bacteriological work of the last fifty years includes many observations bearing on cellular respiration; but there have been few attempts, so far as the writer is aware, to gather these together and formulate them. The text-books on bacteriology do not contain a chapter on bacterial respiration, and the classification of bacteria into strictly aerobic, facultatively aerobic or anaerobic and strictly anaerobic which they contain is based on Pasteur's work. It will be well, therefore, before going further to consider where exactly Pasteur left this subject.

The possibility of life in the absence of oxygen was proved by Pasteur's experimental investigation of the fermentation of sugar solutions by yeast and other fungi such as *Mucor* (1876) quite apart from his discovery of the *Vibrion butyrique* (1861), and it was in the former studies that he mainly elaborated his ideas of anaerobic growth. He considered fermentation and anaerobic growth to be interdependent; that is to say that even where a fluid was exposed to air the occurrence of fermentation depended on the anaerobic conditions in the deeper parts of the liquid from which oxygen had been removed by the stream of escaping CO_2 gas. The essence of fermentation was, of course, the existence of a high ratio of sugar destroyed to that of yeast developed, and he showed that the more strict the anaerobic conditions the higher this ratio was. He considered further that fermentation was characteristic of forms of life with most active respiratory powers, and that they derived the oxygen they required

from complexes in which it was already in combination. The discovery of the *V. butyrique*, besides furnishing an additional proof of Pasteur's contention that life without oxygen was possible, established the altogether new idea of the existence of animalcules, as he called them, incapable of living in the presence of oxygen and tending to die rapidly when oxygen was present. Pasteur states quite clearly that the *V. butyrique* was killed when a current of air was passed through its cultures for 1 or 2 hours. This last point has not always been sufficiently considered in discussing the rationale of anaerobic growth.

The additional knowledge bearing on this subject which has accumulated since Pasteur's time comes mostly from the study of isolated phenomena associated with and bearing on respiration, and it can, therefore, be best reviewed under the following heads :

(1) Reducing activities of bacteria.

(2) Production of peroxide by bacteria and the problem of anaerobiosis.

(3) Oxidizing activities of bacteria—oxidizing ferments and catalase.

(4) The influence of gaseous environment on bacteria.

(5) Production of CO_2 and other gases by bacteria—respiratory quotients.

(6) Production of light by bacteria—ratio of oxygen consumption to development of energy.

(7) The conception of oxidation-reduction potentials and its application to problems of bacterial respiration.

Reducing Activities of Bacteria.

Under the impetus of the work of Wieland (1913, 1921, 1922), Thunberg (1920) and others our outlook on biological oxidations and reductions has widened. It has been realized that methylene blue and other so-called hydrogen acceptors can take the place of oxygen in various oxidations, that they are reduced in the course of the oxidation, and that, therefore, strictly speaking, there is no oxidation without a corresponding reduction whether it be of oxygen or of some more complex substance. The discussion of bacterial oxidations and reductions as if they were independent phenomena is, therefore, impossible ; e.g. the extensive studies of the Cambridge School of biochemists on the dehydrogenating activities of ' resting ' bacteria might be discussed as studies on the reduction of methylene blue on the one hand, or on the other as studies of the oxidation of succinic acid, lactic acid, &c. Those studies of reductions in which only the change in the substance reduced have been considered will, however, be reviewed first.

Reduction of Dyes.

The idea of using reduction of dyes as a means of differentiating between closely allied types of bacteria is an old one. Thus, in differentiating streptococci, v. Lingelsheim (1891) used sodium indigo sulphonate,

Menge and Kroenig (1899) both litmus and indigo, Gordon (1905) and Andrewes and Horder (1906) neutral red, and more recently Sherman and Albus (1918) have returned to litmus and indigo carmine.

The adoption of methylene blue as a substance particularly appropriate for observing bacterial reducing activities led to the conception that all bacteria were endowed with reducing power (Smith, 1896). Although this is probably true, it was emphasized by Müller (1899) that there were qualitative as well as quantitative differences in the reducing powers of bacteria, e.g. that *B. coli* reduced methylene blue twice as quickly as *V. choleræ*, but that the reverse held for litmus. All these indicators of reduction are coloured substances which become colourless when reduced, and tend to resume colour as soon as the reducing action of the bacteria is diminished, either by vigorous aeration of the culture or owing to decreasing vigour of the bacterial growth. Lipschitz (1923) and Bieling (1923[1 & 2]) have introduced indicators showing colour in the reduced state. These are nitrobenzenes or nitroanthraquinones, and since the degree and persistence of their reduction is quite independent of the presence of oxygen they have suggested that the colour developed is a measure of the activity of bacterial respiration and they describe *Meningococcus*, *B. typhosus*, *B. paratyphosus* B and *Pneumococcus* as bacteria which are less active in their respiration.

A point which has been much discussed is whether the dyes are reduced by products secreted by the bacterial cell and elaborated in the medium outside the cell or whether reduction is an intracellular process. Th. Smith (1896) was unable to separate reducing substances from cultures of *B. coli ;* but Müller (1899), Carapelle (1908), Wichern (1910) and others have brought forward evidence to show that metabolic bacterial products can produce a reducing action at a distance from the bacteria. It could not be said, however, that a very convincing proof of the reduction of dyes independently of the bacterial cell had been produced till Avery and Neill (1924) and Neill (1925) demonstrated marked reducing activities in cell-free filtrates obtained from disintegrated pneumococci. Even so the necessity for radical disintegration of the cell before active filtrates could be obtained makes it clear that bacterial reductions normally occur intracellularly or at the cell surface. Th. Smith supposed that it was the intracellular reduction of the dyes, not the impermeability of their surface, which made bacteria insusceptible to vital staining. Gordon and McLeod (1928) and Ellingworth, McLeod and Gordon (1929) point out that even substances like the *p*-phenylenediamines which are actively oxidized to coloured compounds in bacterial colonies do not appear to colour the interior of the cells, and that this may be due to a combination of oxidation at the cell surface and intracellular reduction.

A practical point in regard to the use of indicators in determining fermentation of sugars, &c., by bacteria is that false conclusions may be reached owing to overlooking the reducing activities of the bacteria concerned (Geilinger and Schweizer, 1923).

An interesting observation of Beijerinck (1904) is that the pigment produced by *B. pyocyaneus* is reduced within the body of the bacillus.

Reductions of Various Inorganic Substances.

Nitrates and nitrites. On account of its economic importance for agriculture, bacterial denitrification, i.e. the reduction of these salts by soil bacteria, has been extensively investigated. The outstanding work on the subject is that of Maassen (1902), who showed that the majority of bacteria capable of growing in simple media reduced nitrates to nitrites, that many but not all of these destroy nitrite, and that some can destroy nitrite although incapable of reducing nitrate. The destruction of nitrite usually depends on its conversion to NH_3, which is used by the bacteria immediately it is formed. There is one group, however, the true denitrifying bacteria, *B. fluorescens liquefaciens* and allied forms, which utilize the oxygen of the nitrite and give off nitrogen. It is owing to this character that *B. pyocyaneus* is capable of pseudo-anaerobic growth according to Braun and Cahn-Bronner (1921[1]).

The vibrios are amongst the bacteria which reduce nitrate and not nitrite, hence the accumulation of the latter in their cultures and the nitroso-indole reaction given by those which also produce indole, e.g. *V. choleræ.* Apart from the economic interest of denitrification, bacteria have been differentiated by their actions on nitrates and nitrites. Pelz (1910) showed that bacteria could be split into active, moderately active, slightly active and inactive groups, according to their capacity for reducing nitrate. The vibrios and mannitol-fermenting dysentery strains came into the first class, *B. coli* into the second, and streptococcus into the last. Logie (1910, 1911) showed that non-mannitol-fermenting dysentery bacilli were distinguished from the forms which fermented mannitol by their inability to reduce nitrite. He also pointed out that in a medium containing glucose, HNO_2 may be liberated from sodium nitrite by organic acids formed, and nitrogen be evolved owing to interaction between HNO_2 and the NH_2 groups of the proteins, as in the Van Slyke method for estimation of amino-acids. Under these circumstances there may be a pseudo-nitrite reduction. Orla Jensen (1919) states that the lactic acid bacteria do not reduce nitrate. Anaerobes, which reduce dyes and like substances very readily, fail to reduce nitrates (Gordon, unpublished), and are very poor reducers of potassium tellurite (Gosio, 1905). *B. welchii* appears to be an exception, however.

Sulphates. Most bacteria growing in pure culture fail to reduce sulphates (Sasaki and Otsuka, 1912 ; Bürger, 1914 and Myers, 1920) ; but Salkowski (1913) pointed out that, in the mixed cultures represented by sewage, sulphates were certainly reduced, and Beijerinck (1904) has described two anaerobic spirilla isolated from the mud of canals and harbours which produce H_2S in media containing no other form of combined sulphur than sulphate.

Phosphates. Barrenscheen and Beckh-Widmanstetter (1923) found the literature with regard to bacterial production of PH_3 very conflicting. In their own observations this gas was produced in anaerobic cultures of cadaveric blood, but they failed to isolate any bacterium which would produce this gas when inoculated to sterile blood. Liebert (1927) could get no evidence for reduction of phosphates by bacteria and considers it altogether improbable that such reduction does occur.

Other inorganic substances. Amongst these which have been investigated are selenates and selenites, tellurates and tellurites and ammonium molybdate (Beijerinck, 1904 ; Gosio, 1905 ; Levine and Jahr, 1921). A practical application of the reduction of tellurium salts by bacteria has been made in the recognition of colonies of diphtheroid bacilli by their inability to reduce telluric acid (J. Smith, 1914), and Gosio suggested that a dilute solution of potassium tellurite should be added to all vaccines, &c., in order that blackening should serve as a danger-signal if living bacteria were present.

Reduction of Substances of Biological Importance.

Cystine and glutathione. It was shown by Hopkins (1921) that the thermo-stable substance present in yeast and muscle extracts and capable of giving the nitroprusside reaction in the reduced state was a tetrapeptide consisting of two molecules of glutamic acid and two of cystine in the oxidized state and a dipeptide consisting of one molecule of glutamic acid linked to one molecule of cysteine in the reduced state. Hopkins (1929) has brought forward evidence to show that glutathione is a more complex substance than was till now supposed. When McLeod and Gordon (1924) investigated the capacity of various bacteria to reduce oxidized glutathione, i.e. to produce a positive nitroprusside reaction in meat extract and yeast extract which had been exposed to the air till the original nitroprusside reaction had disappeared, it was found that a much more definite gradation of their reducing activities was demonstrated than could be obtained with methylene blue. They fell into four classes : (1) weak—streptococci and *B. dysenteriæ* (Shiga) ; (2) moderately active—*Staphylococcus* and *Pneumococcus* ; (3) active—the coliform bacteria ; (4) very active—the anaerobes. Evidence for a roughly similar gradation of bacterial activity in reducing cystine to cysteine was obtained by McLeod and Gordon (1925[2]) in the production of haloes of clearing around colonies on an agar medium rendered opaque with excess of undissolved cystine. Since cystine is relatively insoluble and cysteine easily soluble, this result would be expected to follow reduction. The relative importance of reduction and proteolytic activity in the production of H_2S by bacteria growing in protein media is not clear, but there is close association between H_2S production by bacteria and their reducing activity, and it has been shown that bacteria producing H_2S do so more actively if cystine is added to the medium (Sasaki and Otsuka, 1912 ; Bürger, 1914 ; Myers, 1920 ;

Kondo, 1923). In fact, when a medium containing cystine and lead acetate is used and incubation is under anaerobic conditions, even bacteria like the hæmolytic streptococci, which are not usually accepted as producing H_2S, can be shown to produce blackening (Bertha Wheatley, unpublished).

Toxins. A very important contribution to our knowledge of the effect of reduction and oxidation on bacterial products is the recent demonstration by Neill (1926[1, 2, & 3]) and Neill and Mallory (1926) that bacterial hæmolysins, which have long been known to lose their activity rapidly on standing exposed to air, may have it restored by reduction. In so far as streptolysin is concerned this result has been confirmed by Channon and McLeod (1929), but in their experiments the restoration, though definite, was incomplete.

Hæmoglobin and hæmoglobin derivatives. A certain amount of reduction of some of these substances may be caused by the culture media alone ; but in speed and intensity these reductions are much less than those produced by most bacteria. As a reagent for detecting the reducing activities of bacteria, oxyhæmoglobin is much like methylene blue—all bacteria reduce it (v. Liebermann, 1909). Further, Schnabel (1921) has pointed out that in the absence of oxygen *Pneumococcus* will reduce methæmoglobin, although oxidation of hæmoglobin to methæmoglobin is the action of *Pneumococcus* to which most attention has been paid. In the altered blood-pigment present in heated blood-agar—presumably hæmatin —we have a product capable of reduction by bacteria but much less easily reduced than dyes such as methylene blue, and, therefore, suitable for demonstrating the gradation in the reducing activities exerted by different bacteria. With cultures in deep tubes of heated blood-agar all gradations are observed from a slight pink tinge towards the bottom of the tube after several days' incubation of a culture of *Gonococcus* to a frank reddish pink discoloration throughout the whole tube, except for one-eighth of an inch at the air surface, within 12 to 24 hours of the inoculation of a vigorous anaerobe. The marked reducing powers of anaerobic bacteria for hæmatin facilitates the detection of their colonies in plate cultures on ' chocolate agar ', although the effect is a fleeting one and fades in a few minutes on exposure to air. Plates illustrating some of these effects have been published by McLeod and Gordon (1923, 1925[1]) and McLeod (1928). A most interesting suggestion made by v. Liebermann (1909) and with more precision by Dibbelt (1912), is that the cause of death in acute septicæmias, such as those produced in animals by members of the *Pasteurella* group of bacteria, is not intoxication so much as suffocation ; the tissues of the body being unable to compete with the bacteria for the oxygen of the oxyhæmoglobin since the latter is reduced before it reaches them.

Production of Peroxide by Bacteria and the Problem of Anaerobiosis.

There are many experiments recorded in chemical literature to show that whenever active hydrogen is generated in the presence of oxygen there is a tendency to the formation of H_2O_2. Thus Wartenberg and Sieg (1920) showed that H COOH and H_2O_2 both occur in an ice-cooled CO flame and that the former is disintegrated with liberation of CO_2 and H_2, the union of the latter with O_2 being responsible for the production of H_2O_2. Zelinsky and Borissow (1923) demonstrated that when oxygen is passed rapidly through acidulated water containing strips of palladium foil loaded with hydrogen there is a gradual accumulation of H_2O_2 in the fluid, reaching a maximum of 0·6 gm. per litre at the end of 6 hours. Hofman (1923) refers to the production of H_2O_2 by the union of O with activated hydrogen when oxygen and hydrogen are brought into contact with palladium, iridium or platinum finely distributed over porous clay in a weak solution of acid or alkali. There is also evidence that the union of hydrogen and oxygen to form H_2O_2 occurs under the influence of such reducing agents as leuco forms of dyes. Baur (1908) pointed out that H_2O_2 is formed when a solution of indigo white is aerated and can be demonstrated if $Ba(OH_2)$ is added to take up and remove the H_2O_2 from the reacting mixture as it is formed. Lastly the more recent biochemical literature shows that in the course of oxidations in which a dehydrogenating enzyme such as the xanthine oxidase of milk is active there is secondary formation of H_2O_2 (Thurlow, 1925). That is to say that an enzyme capable of transferring hydrogen from the substance on which it acts to methylene blue, or any other hydrogen acceptor, will produce H_2O_2, when oxygen acts as acceptor and no substance very actively catalysing the decomposition of H_2O_2 is present. There is ample evidence in the work of Quastel (1925), Quastel and Whetham (1925[1 & 2]), Quastel and Wooldridge (1925 and 1929) and Stephenson (1928) that bacteria are possessed of such enzymes. Since, therefore, many bacteria produce just such reductions as depend on the intervention of active hydrogen, there is every reason to suppose that they should produce H_2O_2 in the presence of oxygen, and further, that their capacity for producing H_2O_2 should be determined primarily by the activity of their powers of reduction (McLeod and Gordon, 1925[2]). That H_2O_2 production by bacteria remained so long undetected is due to the fact that its accumulation in cultures is controlled by two factors—bacterial catalase and the degree of bacterial sensitiveness to H_2O_2. It was, therefore, natural that it should have been first discovered in cultures of bacteria which are devoid of catalase, only moderately sensitive to H_2O_2, and possessed of a fairly potent reducing mechanism of the type in which active hydrogen is liberated—the pneumococci (McLeod and Govenlock, 1921; McLeod and Gordon, 1922[1 & 2]). This finding has been confirmed by Avery and Morgan (1924[1]) and others.

Other bacteria capable of growing aerobically which have been shown to accumulate traces of peroxide in their cultures are streptococci,

especially the common streptococcus of the mouth, some sarcinæ, *B. bulgaricus* and *B. acidophilus*. The evidence has either been reaction with the usual reagents for detecting H_2O_2, or, in the case of those which produce least, indirect evidence from the blackening of benzidine in blood media, i.e. in media containing peroxidase (McLeod, Gordon and Pyrah, 1923). All these bacteria have in common the absence of catalase, they would be classified by Beijerinck as micro-aerophilic forms and they belong to Orla Jensen's group (1919) of true lactic acid bacteria.

Kluyver (1924) pointed out that bacteria could be divided into two main classes : (1) those possessed of catalase and capable of direct oxidation of foodstuffs at the expense of free oxygen and (2) those existing entirely by fermentative processes independent of free oxygen, the lactic acid bacteria and the anaerobes. In view of this, the discussion of the production of H_2O_2 by the lactic acid bacteria may be followed by the discussion of anaerobiosis.

Anaerobiosis.

The principal problems which have been discussed in connection with anaerobic bacterial growth are three :

(1) Are the anaerobic bacteria completely independent of free oxygen ?

(2) Why are they intolerant of oxygen and what are the limits of their tolerance of that gas ?

(3) What is the nature of the mechanism by which they obtain the energy that they require, in the absence of free oxygen ?

Independence of Free Oxygen.

It was clear from Pasteur's original studies that even facultative anaerobes could flourish in media containing at most infinitesimal traces of free oxygen ; and although it has been sustained, notably by Beijerinck (1904), that anaerobes require traces of free oxygen to develop the energy they need for the reductions which they cause, the weight of experimental evidence has gone to show that they are capable of unlimited propagation in the entire absence of oxygen. This evidence is summed up by the Medical Research Committee (1919), and need not be repeated here. Further, Kürsteiner (1907) claims that this capacity for unlimited sub-culture in the complete absence of oxygen is characteristic of facultative anaerobes as well as strict anaerobes. Quite recently and approaching the subject from a new angle, Clark (1928) and Cohen (1928) have come to the conclusion that the concentration of free oxygen present in cultures of anaerobes must be at most from 10^{-66} to 10^{-80} atmospheres, i.e. nil. Their arguments are based on consideration of oxidation-reduction potentials, to which some reference will be made below.

Beijerinck (1899) contended that anaerobes grew most freely where traces of oxygen were present, and Burri and Kürsteiner (1908) have pointed out that growth is more profuse in anaerobic cultures if they are slightly exposed to air after anaerobic growth has been established. Such findings are not necessarily at variance with those in which the capacity

of anaerobes to live quite independently of oxygen is established. They do not appear, however, to have been subjected to much subsequent investigation.

Intolerance of Oxygen.

There are a number of data in the literature which suggest that anaerobes are by no means equal in the degree of their intolerance of oxygen and the selection of certain anaerobes such as *B. tetani* as test organisms in judging the quality of an apparatus for obtaining strictly anaerobic conditions is good evidence that an inequality in the tolerance of oxygen exhibited by different anaerobes is generally recognized. Pesch and Gottschalk (1924) point out that the diphtheria bacillus will still grow at oxygen tensions which are low enough to permit of slight growth of *B. tetani*, but not when the oxygen tension is low enough to permit of free growth of *B. tetani*. Obviously, therefore, traces of oxygen may be tolerated even by strict anaerobes. Amongst the more exact observations on this subject are those of Chudiakov (1898) who found that butyric acid bacteria would tolerate 5 to 10 mm. pressure of air, *B. tetani* 10 to 20 mm. and *B. chauvœi* 40 mm. Wolf and Harris (1917) describe *B. welchii* as a limited anaerobe, since it grows in fluid cultures through which a current of 1 per cent. oxygen and 99 per cent. nitrogen is bubbled.

In taking up this question afresh the writer was surprised to observe how wide the variation in oxygen tolerance was amongst bacteria described as strict anaerobes. *B. tetani* was found to tolerate 5 to 15 mm. pressure of air when growing in surface culture on heated blood-agar, whereas *B. welchii* grew fairly well at a 200 mm. pressure and grew slightly when the air pressure was half of the ordinary atmospheric pressure. It was suggested by the writer and Gordon (1923[2]) that the intolerance of oxygen characteristic of anaerobes might be explained by their tendency to produce H_2O_2 when brought into contact with air, as it could be shown that their growth was inhibited by the presence of traces of peroxide in media incubated in an atmosphere free from oxygen. It is obvious from what has already been said about the marked reducing activities of anaerobes that it is altogether likely that they would produce H_2O_2 when their cultures were brought into contact with air, and that, being devoid of catalase (Löwenstein, 1903; Rywosch and Rywosch, 1907; Callow, 1923; and McLeod and Gordon, 1923[1]), they would be unable to protect themselves from the H_2O_2 formed. However likely it may be that anaerobes do produce H_2O_2 when in contact with air, the demonstration that they do so has necessarily been difficult, since their active life is inhibited before enough peroxide has accumulated in their cultures to give the usual chemical tests. Observations made with recognized reagents for H_2O_2 on aerated cultures of anaerobes in fluid media have given negative results in the writer's experience as also in that of Callow (1923).

There is, however, evidence of considerable value that anaerobes do produce H_2O_2 in the green discoloration of heated blood-agar which can

be shown in all cultures of anaerobes in deep tubes about $\frac{1}{8}$ to $\frac{1}{4}$ inch below the surface, i.e. at the zone where the penetration of oxygen is held in check by the reducing activity of the bacteria (McLeod and Gordon, 1925[1]). Such colour changes have also been figured by Henry (1917) and described by Heller (1922). A similar discoloration is produced by all bacteria which can be shown by direct tests to be producers of H_2O_2 and its intensity is proportional to their activity as H_2O_2 producers. It has also been shown that where an anaerobe grows at an air pressure just low enough to permit of scanty growth the colonies in surface cultures on heated blood-agar develop green haloes similar to those which surround streptococcal colonies grown under air at atmospheric pressure (McLeod, 1928). Further, surface colonies of *B. botulinus* grown on a heated blood-medium containing benzidine develop a blackish halo within an hour of exposure to air. Finally, it has been shown with an unusual anaerobe obtained on three separate occasions from pathogenic conditions in man that direct tests for H_2O_2 can be obtained if the centrifuged sediment from its cultures in fluid media is vigorously oxygenated for 15 minutes. Hagan (1924) has also described an anaerobic form, an actinomyces, in the cultures of which recognizable amounts of H_2O_2 are formed on aeration.

The actual production of H_2O_2 in aerated cultures of anaerobes has not been directly disputed, but it has been suggested that there is no need to introduce a hypothetical production of H_2O_2 to explain the phenomena of anaerobiosis (Novy, 1925; Quastel and Stephenson, 1926). Novy is probably right when he maintains that the fresh potato which promoted the growth of anaerobes in Avery and Morgan's (1924[2]) experiments did so because it absorbed oxygen, and not because it destroyed toxic peroxides, and Quastel and Stephenson are probably also right when they attribute the value of cysteine in promoting the growth of *B. sporogenes* in their experiments to its power of neutralizing oxygen in the culture fluid and establishing a definite reduction potential.

There is no doubt that from the time of Tarozzi's work (1905) onwards the various experiments for obtaining growth of anaerobes in fluid media exposed to air have depended on absorption of oxygen within the medium; such are the use of (1) soluble ferrous salts (Guillemard, 1911): (2) pyruvic acid (Berthelot, 1923): (3) Na_2S, originally suggested by Trenkmann and reintroduced by Manteufel (1922): (4) artificial peroxydase prepared from gum arabic and $FeSO_4$ (Avery and Morgan, 1923, 1924): (5) sodium pentacyanoaquaferroate (Webster and Baudisch, 1925). Kodama (1926), however, has shown that the effect of traces of H_2O_2 in destroying a reducing potential is much greater than that of oxygen, and, if oxygen is excluded or masked, peroxide production will also be suppressed. Besides this it has been overlooked by Novy that a theory of anaerobiosis has not only to explain the conditions in which anaerobes will live, but also the rapidity with which they die when exposed to air. Quastel and Stephenson have not overlooked this point but they have dealt with it

inadequately. They have shown that a culture of *B. sporogenes* in a fluid medium is not at all easily killed off by vigorous aeration, and conclude that H_2O_2 production by anaerobes is not an important factor in anaerobiosis. The weak points in this argument are: first, that the anaerobe most reputed for its powers of resistance and for the rapidity with which it spores was chosen for these experiments; secondly, that they did not choose the conditions in which bacteria are most completely exposed to oxygen, namely, spreading on a surface; and thirdly, that they made no attempt to determine how many of the bacteria present at the outset of their aeration were alive when it was finished.

There is a considerable body of evidence to show that anaerobes in their vegetative forms die very rapidly when exposed to air, and that some even in their sporing form do not resist aeration long, although in the case of others the resistance of the spores is remarkable. Chudiakov (1898) found that vegetative forms of a butyric acid bacillus exposed in gelatin plates had mostly died off in the first 4 hours of exposure, although all were not dead till 15 hours had elapsed. Lüderitz (1889), working with spore-free cultures of an anaerobic bacillus from the soil, got similar results. Bachmann (1912) noted that the great majority of anaerobic forms were killed after a short period of exposure, and he is inclined to suppose that the minority which are not killed off so readily are sporing forms in an early stage of development at which spores are not recognizable. He inclines to the idea that a toxic product is formed in cultures of anaerobes exposed to air and that this is responsible for the death of the vegetative forms. The difficulty which is often experienced in sub-culturing from the spreading margin of very young cultures of *B. tetani*, i.e. from a spore-free area, is not easily explained except in the way suggested by Bachmann. The vegetative forms appear to be often destroyed by the briefest exposure to air.

In the experience of the writer young cultures of *B. sporogenes* which have been incubated in a medium specially suitable for growth and from which oxygen has been completely excluded, and which are therefore composed largely or entirely of non-sporing forms, are found to be extremely susceptible to oxygen. If equal inocula of such cultures are made to a series of agar tubes, which are then poured as plates and brought under anaerobic incubation at varying intervals of time, it is found that, although the colonies are innumerable in the plate which is brought under anaerobic conditions as soon as it has set, there are very few or none in the plates exposed for 1 to 3 hours before being brought into conditions of anaerobic incubation. It is obvious that this result is not due to accumulation of oxygen in the plate since plates poured at the same time, and inoculated just before incubation grow *B. sporogenes* quite freely. It is difficult to come to any other conclusion in view of these results than that a lethal product is formed owing to the effect of oxygen on the bacteria or on some product of their metabolism. There is much evidence for supposing that this product is H_2O_2.

The Nature of the Process by which Anaerobes Obtain the Energy which they Require.

Although there is evidence (Braun and Cahn-Bronner, 1921[1]) to show that aerobes like *B. pyocyaneus* can actually live in the absence of free oxygen by utilizing the combined oxygen of nitrates as if it were atmospheric oxygen, there is no reason to suppose that anaerobes get their energy in this way. They would seem rather to disintegrate compounds by withdrawal of hydrogen and to derive energy from the exothermic reactions which they thus set up, and it is precisely because the energy available in this way is so small compared with that obtained by oxidation of the same molecules at the expense of oxygen that anaerobic bacteria disintegrate such large quantities of carbohydrate and protein in the course of their activities. Stephenson and Whetham (1924) observed that glucose, which can be decomposed with liberation of energy in the absence of oxygen, will promote the anaerobic growth of *B. coli*, whereas succinic and lactic acid, which will only yield energy on oxidation by oxygen, are incapable of doing so.

The necessity for more complex media for the anaerobic growth of facultative anaerobes capable of growing on extremely simple synthetic media when allowed access to air had already been pointed out by Braun and Cahn-Bronner (1921[2]). Quastel and Stephenson (1925) have elaborated a theory of anaerobic growth, according to which it is dependent on the existence in the medium of substances which will act as acceptors of hydrogen and others of which the hydrogen is activated. The latter are oxidized by the transfer of their hydrogen to the acceptors; a further necessary condition is that the product of oxidation should be capable of being built up into the bacterial protoplasm. Quastel and Stephenson were able to bring most, but not all, of their experimental observations into line with this scheme. It is interesting that partial poisoning with KCN (Burnet, 1927), H_2S (Negelein, 1925) and certain degrees of heat (Ørskov, 1925) will convert facultative anaerobes into obligatory anaerobes. The group of true lactic acid bacteria and strict anaerobes—the catalase-deficient bacteria—are indifferent to KCN in the concentrations in which it produces this result (Burnet, 1927).

Before leaving the subject of the anaerobes it may be noted that Sherman (1921, 1926) has described the propionic acid bacilli as anaerobes endowed with catalase activity. Through the kindness of Dr. Sherman the writer obtained a strain of this bacillus from America and compared it with a strain freshly isolated from Gruyère cheese. The two strains resembled each other closely and showed the characters described for this type of bacterium, with the exception that they invariably grew scantily but quite definitely on heated blood-agar containing sodium lactate and freely exposed to air at atmospheric pressure. They cannot, therefore, be considered as an exception to the characteristics of anaerobes as described above (Dr. F. C. Happold, unpublished).

Oxidizing Activities of Bacteria, Oxidizing Ferments, Catalase.

These activities can be divided into two categories : (1) those to which reference has already been made depending on a ferment of the nature of a dehydrogenase, and (2) those depending on the participation of free oxygen and probably associated with iron catalysts in the sense of Warburg (1914, 1923).

Oxidations by Dehydrogenating Ferments.

Much the most extensive work on this subject has been done by the Cambridge school of biochemists. The essential points in these experiments are that washed bacteria are suspended in buffer solution, persisting traces of oxidizable matter are removed by passing an air current for some time, and then the suspension is mixed with a dilute solution of methylene blue and a 1 per cent. solution of some substance to be investigated. The mixture is kept at 45° C. in a vacuum tube which has been exhausted. Most of the experiments were carried out with *B. coli*. Under these conditions, succinic acid was converted to fumaric (an oxidation), while, simultaneously, methylene blue was converted to its leuco compound (a reduction). If fumaric acid was substituted for succinic the converse of this process took place, i.e. methylene blue was formed by oxidation of its leuco compound and fumaric acid was reduced to succinic. In other words the bacteria possessed a dehydrogenating enzyme or dehydrogenating enzymes capable of oxidizing both succinic acid and methylene blue. Further, these processes were not hindered by concentrations of KCN sufficient to arrest oxidation processes dependent on the utilization of free oxygen through the agency of catalysts containing iron. The oxygen uptake of preparations of ' resting bacteria ' with succinate and fumarate respectively, however, were found to be similar. It was obvious, therefore, that a further oxidation of the fumarate was only possible in the presence of oxygen. Later work by Stephenson (1928) has shown that a cell-free enzyme prepared from *B. coli* dehydrogenates lactates with production of pyruvates, while the further oxidation of pyruvates which can be obtained with access of oxygen is dependent on the presence of the bacterial cell, and is, unlike the former, sensitive to KCN. In its indifference to KCN the bacterial dehydrogenase resembles the xanthine oxydase of milk, which is obviously an enzyme of the dehydrogenase type (Dixon and Thurlow, 1925).

These methods have been used, therefore, to determine the extent to which different substances are capable of anaerobic oxidation by bacteria. The presence of certain sugars—notably glucose and lævulose—much increased the rapidity with which methylene blue was reduced by bacteria. Other substances were less active. Certain fatty acids most nearly approached the sugars ; the order of effectiveness of the most potent being formic acid, lactic acid and succinic acid, and it was found that amino-acids were surprisingly inactive (Quastel and Whetham, 1925[1 & 2]).

It is to be noted that Oberstadt (1913) with some variations in technique had covered a good deal of this ground and reached very similar results, and that Bieling (1923[2]), studying the stimulating power exercised by various additions to the media on the reduction of nitroanthraquinone by bacteria, found that tissue extracts and some fatty acids—notably succinic—stimulated reduction, while none of a large number of amino-acids tested had any effect. Löffler and Rigler (1926) have applied a technique essentially similar to that of Quastel and his collaborators to the study of the capacity of bacteria to attack sugars, and claim that it is a more delicate method than the usual one for determining sugar fermentation, and that it reveals some unexpected facts, e.g. that Shiga dysentery bacilli are capable of attacking maltose and disintegrate glucose as rapidly as does *B. coli*, and that *B. fæcalis alkaligenes* is capable of attacking sugars, although feebly. Further, Quastel (1928) points out how valuable such methods may be in detecting intermediate products of metabolism which would escape observation in cultures of proliferating bacteria, because of the speed with which they were carried to a further stage of oxidation.

In some of these experiments (Löffler and Rigler, 1926) it is assumed that observations of the reduction of methylene blue is tantamount to measurement of oxygen uptake, but this can only be true in a limited sense. How marked this divergence may be is emphasized by the work of Cook and Stephenson (1928), in which the uptake of oxygen with acetate and formate were approximately equal, whereas, compared with regard to their capacity to accelerate the reduction of methylene blue by resting *B. coli*, formate was found seven hundred times superior to acetate. Callow (1924) noted that most washed bacteria when suspended in buffer solution and observed in a Barcroft apparatus, showed an oxygen uptake of 5 to 25 c.cm. per gm. dry weight per hour and that *B. subtilis* was specially active. *B. sporogenes* and *Streptococcus acidi lactici*, the two members of the group of catalase-deficient bacteria examined, caused a negligible absorption of oxygen. It is interesting in this connection that although Burnet (1927) got a similar result when working with ' resting bacteria ', he found that the distinction between oxygen-absorbing and certain non-oxygen-absorbing bacteria might be obliterated when he worked with cultures. It would seem, therefore, that, valuable as the results of this work with ' resting bacteria ' undoubtedly are, there are still difficulties in their interpretation.

The Catalysis of Bacterial Oxidation requiring Free Oxygen.

When the biological catalysis of oxidation was written up about the beginning of this century, e.g. by Enriquez and Sicard (1902), three types of substance were considered : (*a*) direct oxidizing ferments ; (*b*) indirect oxidizing ferments, i.e. such as required the presence of a peroxide before they would exert their action ; (*c*) oxidizing bodies, i.e. bodies which acted like ' b ', but being thermostable could not be considered as ferments.

Kastle (1910) reviewing the subject a little later takes into consideration the action of catalase as well. To these older conceptions we have to add the more recent ones : that of Warburg (1914), that a certain integrity of cell surface in which lecithin and traces of iron are present is of special importance in oxidation ; and that of Hopkins (1920), that organic compounds of sulphur, equipped with a free sulphydryl (SH) group in the reduced state are of special importance in catalyzing oxidations by acting as intermediaries for the transfer of hydrogen to oxygen, as well as being capable of promoting secondary oxidations. An example of the latter is the secondary oxidation of nitrites to nitrates in the course of autoxidation of cysteine (Thurlow, 1925). It is probable that H_2O_2 is formed in course of this reaction but it has not been detected. Warburg and Sakuma (1923), however, have emphasized the importance of traces of iron present as impurity in promoting the autoxidation of cysteine, and it could, therefore, be supposed that a complex of iron and cysteine functioned as peroxidase with the H_2O_2 believed to be produced in the course of the reaction. How far then have these various systems for catalysing oxidation been recognized in the bacterial cell ?

Oxidases and Peroxidases.

Raper (1928), in a recent review of the aerobic oxidases, defines them as ferments which carry out oxidations that cannot be repeated with a dehydrogenating system such as active palladium and methylene blue from which oxygen is excluded. There are four or five ferments described in this category. *Peroxidase,* which has been prepared in a relatively pure state and of which the activity is estimated by oxidation of pyrogallol to purpurogallin in the presence of a fixed amount of H_2O_2. *Polyphenolase,* which with an addition of H_2O_2 oxidizes phenols with two OH or NH_2 groups in the molecule. It is suggested by Szent-Györgyi (1925) that preliminary oxidation of catechol or allied substances to quinone furnishes a body of high oxidizing potency that gives various oxidations characteristics of these oxidases. *Dopa oxidase* which produces melanin from 3 : 4 dihydroxyphenylalanine. *Indophenol oxidase* which produces indophenol when acting on mixtures of α-naphthol and *p*-phenylenediamines. And, lastly, *tyrosinase* which oxidises tyrosine to coloured compounds.

Peroxidase. Kirchner and Nagell (1926) attempted to determine bacterial peroxidase by the classical method of oxidation of pyrogallol in presence of H_2O_2, but they found that catalase effects interfered with their observations.

Tyrosinase. Lehman and Sano (1908) observed the development of brown discoloration of agar around the colonies of three species of bacterium, and concluded that tyrosinase was present, but attempts to extract the ferment were abortive. Beijerinck (1911) claims to have demonstrated this ferment in certain vibrios from sea-water.

Indophenol oxidase and phenolase. It is in regard to this reaction that most work on bacterial oxidases has been done. Dietrich and Liebermeister (1902) described granules in several bacteria which gave this reaction,

notably in *B. anthracis*. This work was not followed up, however, till Schultze (1910) showed that the reaction could be got in a striking way if certain types of bacteria were stroked over the surface of plates in which the reagents α-naphthol and dimethyl *p*-phenylenediamine were incorporated. Kramer (1912) and more recently Nishibe (1926) have extended this work, and Gordon and McLeod (1928) have shown that α-naphthol may be dropped out and the reaction obtained by simply pouring solutions of the phenylenediamines on to plates and off again. The principal point elicited by these investigations is that the oxidative process depends on a thermo-labile system which is present in a number of different species of bacteria, all of which are possessed of catalase and incapable of growing freely in the absence of oxygen.

Catalase.

The exact part taken by catalase in processes of oxidation has not yet been completely determined, but it is known to be closely associated with all oxidations in which free oxygen plays a part, and it is most probable that its function is to eliminate excess of H_2O_2 or even perhaps to maintain a constant low level of H_2O_2. A recent and extensive survey of our knowledge with regard to catalase has been made by Morgulis (1924). Attention appears to have been drawn independently to the catalase activity of bacteria by Gottstein and Beijerinck in 1893. It has since been studied by Jorns (1908), Rywosch and Rywosch (1907) Bujwid (1916), McLeod and Gordon (1923[1]), Schlunk (1924) and others. The absence of catalase in bacteria dependent on anaerobic fermentations and in strict anaerobes has already been referred to. Apart from this the chief facts of interest in regard to it are that it is particularly abundant in pigmented bacteria and in two pathogenic forms—*Gonococcus* and the *B. pertussis ;* that the vibrios are peculiar in showing very slight catalase activity, and that Shiga dysentery bacilli and some streptococci occupy a peculiar position in being devoid of catalase, growing in presence of oxygen and producing no detectable traces of peroxide. Jorns (1908) distinguished between ecto- and endocatalase, finding that filtrates from young cultures of some catalase-active bacteria are almost devoid of catalase, whereas those from bacteria such as *B. pyocyaneus* give an active catalase effect.

Thermo-stable Bodies giving Peroxidase Reactions.

Webster (1924–5) described the existence of 'rough', relatively avirulent and ' smooth ', virulent strains of a *Pasteurella*, the former being richer in peroxidase than the latter ; the method of determining peroxidase is not stated however. Keilin (1925) described a most interesting respiratory pigment allied to, but distinct from, hæmoglobin, easily poisoned by KCN, and one which he had never detected in tissue which did not give a peroxidase reaction. This was found to be present in yeast and in *B. subtilis*, but not in *B. sporogenes*. Callow (1926) described a thermo-stable bacterial peroxidase detected in all bacteria examined, but very weak in *B. sporogenes* and *S. acidi lactici*.

Glutathione.

It was assumed in Hopkins' first publication on this subject (1921) that this system was present in bacteria as in other forms of life. McLeod and Gordon (1924) were, however, unable to confirm this, although a nitro-prusside reaction due to bacterial reduction of the glutathione derived from meat extract was obtained in most cultures. Callow and Robinson (1925) obtained a nitro-prusside reaction with some bacteria, but were unable to obtain it in others. A complete identification of the substance giving this reaction with glutathione does not appear to have been made.

Aerobic Bacterial Oxidations.

The most striking of the bacterial oxidations are those carried through by the so-called autotrophic bacteria—the oxidation of ammonia, nitrite, sulphur and iron (Winogradsky, 1922). Ruhland (1922) maintains that such oxidations are due to iron salts acting as surface catalysts. Unfortunately we lack information on the catalase-, oxidase- and H_2O_2-forming activities of these bacteria. They are interesting on account of their extreme independence of any complex form of organic substance for their nutrition. The work of Braun and Cahn-Bronner (1921[2]) shows that many bacteria usually grown on very complex media can also live on media containing nothing more complex than lactic acid, provided the supply of oxygen is plentiful. Another point of interest in connection with aerobic bacterial oxidations was first emphasized by Ayers and Rupp (1918), namely, the development of alkaline reactions in cultures of some bacteria owing to oxidation of the salts of the fatty acids, with escape of CO_2 and persistence of the base. This type of oxidation was also noted by Stephenson and Whetham (1924), and has been studied by Sierakowski (1923, 1924) and Abt and Loiseau (1925). It is shown in a very striking way by bacteria like *Meningococcus*, which give a marked phenolase test (Phelon, Duthie and McLeod, 1927). Other oxidations of special interest in medicine are those of hæmoglobin and its derivatives, e.g. the methæmoglobin formation observed with extracts of *Pneumococcus* by Neill and Avery (1924), assumed to occur in pneumonia by Peabody (1913) and Stadie (1921), and noted in Gärtner septicæmia in the rat by Boycott (1911) ; and the oxidations of bacterial toxins (Neill), to which reference has already been made.

Production of CO_2 and other Gases by Bacteria ; Respiratory Quotients.

The only gases which are commonly evolved from bacterial cultures, and in regard to which much information is available are H_2, CO_2 and NH_3. Keyes and Gillespie (1912–13), studying the yield of CO_2 per gm. of sugar utilized by bacteria growing under anaerobic conditions found for *B. typhosus*, 2 c.cm. ; *B. coli*, 30 c.cm. ; and *B. welchii*, 74 c.cm. These figures suggest that gas is evolved by bacteria, e.g. *B. typhosus*, which owe their position in their group classification to the fact that they do not produce gas. It would appear, therefore, that the rough methods

of ordinary bacteriological routine allow slight gas formation to escape detection. This is borne out by Ayers, Rupp and Mudge's (1921) observation that special methods determine production of CO_2 by streptococci and by Abt (1925) and Abt and Loiseau's (1925) observation that appropriate measures demonstrate considerable evolution of CO_2 from large bulks of fluid cultures of diphtheria bacilli. Phelon, Duthie and McLeod (1927) made the same observation with *Meningococcus*.

It is obvious that bacteria splitting sugars under anaerobic conditions will show respiratory quotients widely diverging from those associated with oxidation in the animal body. Keys and Gillespie (1912–13) actually observed quotients $\frac{CO_2 \text{ evolved}}{\text{oxygen absorbed}}$ of about 7 for *B. coli* and about 70 for *B. welchii*. Much the most careful work on this question of gaseous exchange by bacteria has been done by Novy, Roehm and Soule (1925), who have devised a very elaborate technique. They record quotients below 1 for protozoa growing fully exposed to air on the surface of culture media, and Soule (1928) points out how quotients above 1 may be produced owing to processes of decarboxylation even when bacteria are oxidizing protein. He suggests further that the $\frac{CO_2}{H_2}$ ratio, which is low for *B. welchii* and high for *B. sporogenes*, may afford a basis for differèntiations amongst anaerobes.

The Influence of Gaseous Environment on Bacteria.

Oxygen. The relationships of anaerobes to oxygen have already been considered; but there are interesting observations on the relations of other bacteria to this gas. Berghaus (1907) determined the maximum pressure of oxygen permitting growth of various bacteria, and the pressure necessary to kill them. *Staphylococcus* was the organism least readily inhibited in its growth by increased oxygen pressure, being capable of tolerating three atmospheres. *B. fæcalis alkaligenes, B. dysenteriæ* (Shiga), *B. pyocyaneus* and *V. choleræ* were most sensitive in the arrest of their growth, but there was a wide divergence in the pressure of oxygen required to kill; for *V. choleræ*, 2 atmospheres, and for *B. pyocyaneus*, 75 atmospheres. Süpfle (1910) found 26·5 per cent. the optimum concentration for the growth of *V. choleræ*. Moore and Williams (1911) tested a considerable number of bacteria but only found *B. pestis*, tubercle bacilli, and *Staphylococcus* sensitive to increased concentration of oxygen in the air. Finch (1913) found tubercle bacilli and 'aurococcus' (? *Staphylococcus aureus*) sensitive to high partial pressure of oxygen, and Karsner, Brittingham and Richardson (1923), observing effects of partial oxygen pressures between 80 and 99 per cent., found *Staphylococcus aureus, Streptococcus, B. coli* and other bacteria sensitive; but *B. pyocyaneus, B. subtilis* and *Pneumococcus* indifferent. Owing to the considerable divergence in these results it is difficult to make any deduction from them.

Carbon dioxide. There is a good deal of evidence to show that the presence of CO_2 favours the growth of some bacteria. A number of observations of this kind depend on the effect of CO_2 in counteracting a tendency to production of excess of alkali by the bacteria (Gates, 1919; Kohman, 1919), and Valley and Rettger (1927) have shown that where the effect of CO_2 on reaction is eliminated the bacteria are tolerant of a very high partial pressure of CO_2, and further, that complete removal of CO_2 inhibits bacterial growth, although the thoroughness with which it must be removed before growth ceases varies considerably with different bacterial species. They also showed that slight increases in atmospheric CO_2 were associated with increased growth of most bacteria, notably *B. acidophilus* and *Br. abortus*. The work of Rockwell (1921, 1923) also demonstrates the importance of CO_2. He points out that some anaerobes which grow well in an atmosphere of hydrogen fail to grow in air absorbed by KOH and pyrogallol: this he attributes to absorption of CO_2 by the alkali in the latter case. He gives a list of various bacteria, including aerobic, anaerobic and facultatively aerobic or anaerobic forms, all of which will grow in a mixed current of nitrogen and CO_2 but not in a current of nitrogen only.

Hydrogen. Berghaus (1907) points out that bacteria are unaffected in their growth by pressures of hydrogen as high as 60 to 70 atmospheres.

Production of Light by Bacteria—Ratio of Oxygen Consumption to Development of Energy.

The bacteria which produce light are for the most part found in the sea, free or on the surface of fish, cephalopods, &c. Many varieties have been described, but the commonest type is a vibrio (Mortara, 1924). Their luminescence is altogether dependent on the presence of free oxygen, although the amount required for luminescence is extremely minute. Harvey and Morrison (1923) calculated that seven parts oxygen in a million were sufficient to enable these bacteria to show visible radiation, and Kürsteiner (1907) adopted these bacteria as indicators of complete removal of oxygen from apparatus to be used for anaerobic culture. Gerretsen (1916) showed that emanation of light continued for several hours after exposure to ultraviolet light which killed off the bacteria, and concluded that the production of light must depend on an enzymatic process.

Harvey (1926[1 & 2]), however, was unable to get any evidence of the existence of a luciferin-luciferase system in luminous bacteria. Such systems consist of a thermo-stable extract in itself non-luminous—luciferin—and a thermo-labile and luminous extract which has lost its luminosity on standing—luciferase. These emit light when brought together, and have been demonstrated in ostracods and fire-flies. The mechanism of the reaction remains obscure. It has been shown by McDermott (1913) that the products of alkaline hydrolysis of peptone show luminescence in presence of aldehyde and H_2O_2, and Gerretsen (1916) has pointed out that all media suitable for the growth of light bacteria contain substances

intermediate between peptones and amino-acids which emit light when boiled with KOH and then exposed after cooling to bromine water. In view of these results and the fact that oxygen is essential to luminescence, it would seem possible that H_2O_2 plays a part in these phenomena. A light vibrio put at the disposal of the writer through the kindness of Prof. J. Cruickshank was found to give a moderately active catalase effect, but did not oxidize the phenylenediamines. If an oxidizing ferment is therefore responsible for light production it is one distinct from those characteristic of many aerobic bacteria. Harvey (1925) points out that light production is not specially sensitive to KCN, which suggests that a peroxidase is not essential to the reaction.

Harvey (1925, 1926[2]) investigated the luminous efficiency of these bacteria and found it very high in the sense that almost all the radiant energy given off is in the form of light. Barnard (1902) had found the light emitted by *Photobacterium indicum* confined to a small part of the visible spectrum, and Forsyth (1910) describes it as extending from 3,500 to 5,000 A.U. with a specially bright band at 4,000 A.U. ; but he considers that the spectrum is more extensive than Barnard had supposed, including rays in the ultraviolet. Guyot (1928) points out that the varieties of light described by Beijerinck probably depend on variations in the media rather than on varieties of bacteria. Harvey (1928), estimating oxygen consumption by the time required for bacteria to cease emitting light in sea-water saturated with oxygen but protected from further access of air, found that it corresponded to 17·46 mgm. oxygen per gm. dried bacteria per hour. Assuming that all this oxygen was used to oxidize protein and other constituents of the medium, he obtained a figure for combustion calories available for bacteria. When this was compared with the caloric value of the energy emitted as light, a minimal energy coefficient of 0·15 per cent. was determined. If, however, it was taken into account that a M/5,000 solution of KCN cut down the consumption of oxygen five times as much as it did the emission of light, it was reasonable to suppose that four-fifths of the oxygen at all events was used for purposes other than generation of light, i.e. the energy coefficient for ratio of light production to fuel burned should be raised five times or to 0·75 per cent. That is to say that the efficiency of luminous bacteria would be about twice that of the best electric bulb.

Waksman and Starkey (1923), studying autotrophic sulphur bacteria, found that the assimilation of 1 gram molecule of CO_2 required the energy derived from combustion of 381·48 gm. sulphur, i.e. 6·6 per cent. of available energy was used.

The Conception of Oxidation-Reduction Potentials and its Application to Problems of Bacterial Respiration.

As the result of ideas and investigations first recorded by Gillespie (1920) and extended by Clark and his collaborators a new precision and form has been given to our methods of measuring oxidizing and reducing

intensities in chemical and biological systems. It is clear from reading Cannan, Cohen and Clark (1926) on 'reduction potentials in cell suspensions' that there are many anomalies in such observations, of which the explanation eludes even those versed in physical chemistry and practised in this type of observation, and obviously the whole subject must be one of great difficulty for those whose training is mainly biological. The conceptions evolved from the bulk of the evidence available do, however, illuminate the data already accumulated with regard to bacterial respiration and must therefore be stated here as simply as possible. Clark (1928) has recently summarized this work and the ideas have been restated by Needham and Needham (1926–7), Aubel and Genevois (1928), Cohen, Chambers and Reznikoff (1928) and Fildes (1929), and to these papers readers are referred for more detailed information than the following brief account.

Since very little is known about single electrode potentials, the practice is to observe the difference in potential between two electrodes. For systemic treatment the potential of some electrode is arbitrarily selected as zero. Then the E.M.F. of a cell made up of this standard electrode and the electrode of which the potential is to be determined is attributed wholly to the second electrode. The normal hydrogen electrode —platinum loaded with hydrogen and immersed in a solution normal in H ion—has been selected as the standard reference electrode having zero potential. It is more convenient, however, to work with a standard electrode such as the saturated calomel electrode which is known to give a potential of + 0·25 volts when connected with the normal hydrogen electrode. This potential is indicated by the expression eH, i.e. the potential of an electrode referred to that of the normal hydrogen electrode.

When an electrode of a noble metal is immersed in a solution of a substance capable of reversible oxidation and in which the proportions of the oxidized and reduced forms are such as to set up a potential equal to that of the normal hydrogen electrode, and when such an electrode is connected with a saturated calomel electrode, an E.M.F. of 0·25 volts will be set up and the sign of the noble metal will be negative. If the substance in solution, the degree of its reduction or the pH of the fluid is varied the observed E.M.F. will vary and with it the eH, the eH being always E.M.F. + 0·25 volts, as indicated below:

E.M.F. observed.	eH.
+0·1	+0·35
0·0	+0·25
−0·1	+0·15
−0·25	0·00
−0·5	−0·25

The point specially emphasized by these observations is that reducing and oxidizing activities are entirely relative, and that a system with a given eH value, whether negative or positive, will oxidize any other

system which develops an eH more negative or less positive and will reduce any system which develops an eH more positive or less negative than its own.

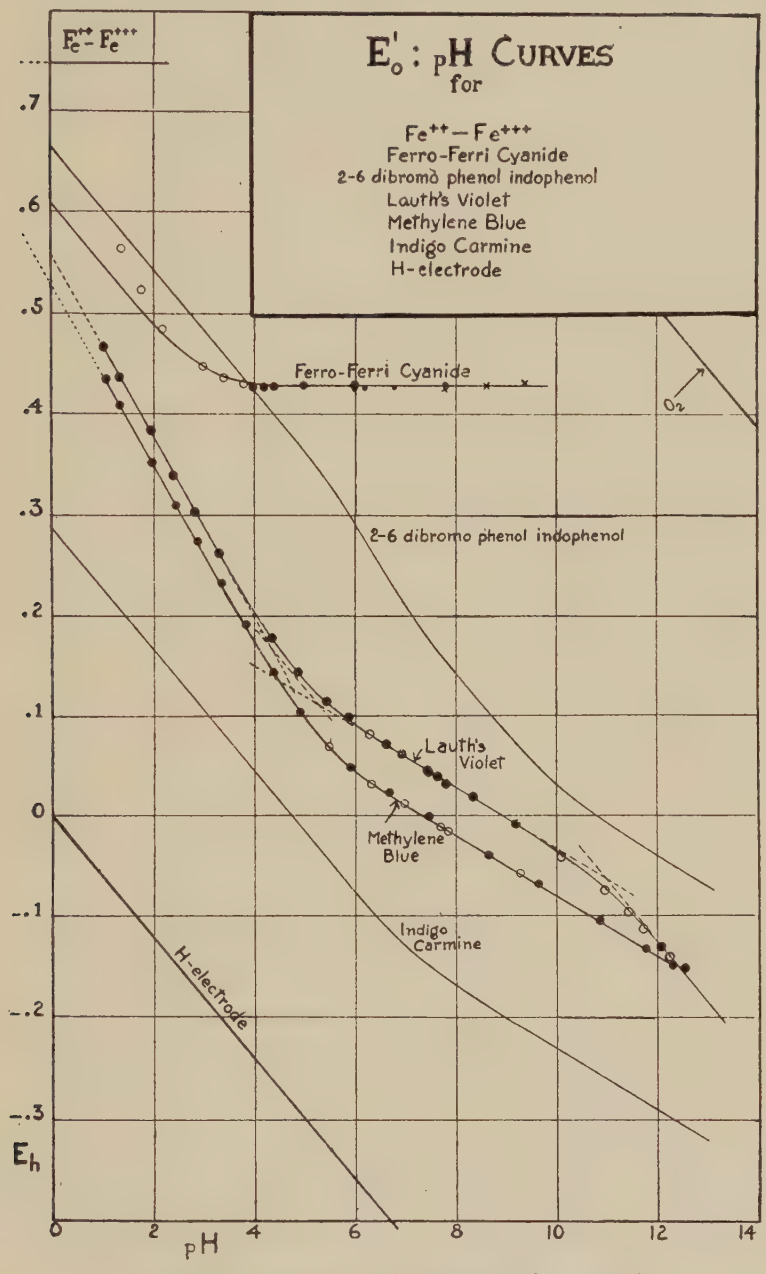
An rH scale has been introduced corresponding to the pH scale, and in this the numbers indicate different reduction potentials ; zero on the rH scale indicates a reducing intensity equal to that of one atmosphere of hydrogen activated under the influence of a catalyst—the platinum electrode—or in other words the reducing activity of the hydrogen electrode ; 6 indicates a reducing intensity corresponding to 10^{-6} of an atmosphere of hydrogen under the same conditions and so on. The advantage of this rH system is that it affords a constant basis of comparison between various reducing systems, and this cannot be derived from the electrical potentials without taking pH into account. That the E.M.F. set up in a system capable of reversible oxidation and reduction like quinone and hydroquinone varies with the pH has been recognized for a number of years, and is actually the basis of all measurements by the quinhydrone electrode (Bilmann, 1921). Thus, the E.M.F. of the hydrogen electrode, which at pH 0 is taken as zero, becomes −0·42 at a pH 7. The influence of pH on eH is shown in the diagram, for the hydrogen electrode, for mixtures of ferro- and ferricyanides and for several dyes present as equimolecular mixtures of the reduced and oxidized forms. This figure has been borrowed by the kind permission of Dr. Mansfield Clark from his study of methylene blue with Gibbs and Cohen (1925).

An extremely useful contribution to these studies has been the introduction by Clark of a number of indicators—dyes capable of reversible oxidation and reduction—which change tint at different parts of the rH scale. Each passes from 100 per cent. of the oxidized form to 100 per cent. of the reduced form within a relatively narrow zone of this scale ; for example, methylene blue at pH 7 and 50 per cent. reduced sets up an E.M.F. of +0·011 volts corresponding approximately to an rH of 14 ; 99 per cent. reduced it sets up an E.M.F. of −0·049 volts corresponding approximately to rH 11 ; 99 per cent. oxidized it sets up an E.M.F. of +0·071 volts corresponding approximately to an rH of 16. Figures showing the relationship of E.M.F. and rH for 3 dyes all 50 per cent. reduced, and at pH 7, which are taken from Cohen, Chambers and Reznikoff (1928), are compared with the potentials of the hydrogen electrode and a strong oxidant in Table I.

TABLE I.

Indicator, &c.	E.M.F.	rH.
Ferricyanide (50 per cent. reduced)	+0·43	28·4
Phenolindo-2 : 6-dichlorophenol	+0·217	21·3
Methylene blue chloride	+0·011	14·4
Potassium indigo disulphonate	−0·125	9·9
Hydrogen electrode	−0·421	0·0

Both the electrometric and the indicator methods have been applied to determine the rH of bacterial cultures. Gillespie (1920) and Cannan,



(From Clark, Cohen and Gibbs, 1928, p. 197.)

Cohen and Clark (1926) have made electrometric observations showing that all cultures investigated may develop potentials sufficiently negative

to effect the complete reduction of methylene blue, but that there is a marked gradation in the potentials developed by different bacteria. Thus, the potential developed in cultures of *B. coli* may approach or even fall below that of the hydrogen electrode, whereas in cultures of *B. subtilis* a negative potential is developed slowly, and barely reaches −0·1 volt. Very similar observations had already been made by Potter (1911, 1912) in this country, who had observed the E.M.F. developed when electrodes dipping into inoculated and uninoculated portions of a culture medium were connected. Potter, however, had not appreciated the bearing of his observations on phenomena of oxidation and reduction.

Cohen (1928) has extended these observations to anaerobes. These are found to give markedly negative potentials. Such findings strongly support the conclusion stated earlier in this paper (pp. 265–8), and arrived at by less exact methods; that is to say that, although Th. Smith's observation that all bacteria reduced methylene blue was correct, the inference that they were equivalent in their reducing powers was erroneous, since observations on their capacity to reduce cystine, hæmatin, &c., ranged them much in the same order of reducing intensity as the electrometric method does.

Another idea developed as a result of this work is that a certain level of oxidation-reduction potential is essential for the development of different micro-organisms. Working with indicators in deep tubes of glucose agar Aubel and Aubertin (1927) noted that anaerobes only developed in parts of a medium in which an rH 12·5 existed. Fildes (1929), in more exact observation on the level of O.R potential suitable for germination of tetanus spores found that the upper limit for the germination of spores at pH 7·0 was the zone of complete reduction of thionin, i.e. +0·01 volt, or an rH about 14. Quastel and Wooldridge (1929), working with *B. coli* grown under anaerobic conditions on a synthetic medium and adding various reducing agents, were unable to convince themselves that an optimum O.R potential for the growth of this bacterium existed. The work of Burnet (1925), however, shows the favouring effect of the vicinity of a colony of *Staphylococcus* on the development of organisms on a plate lightly charged with H_2O_2 by exposure to light. The most likely interpretation of this is that a certain minimum of reduction potential is necessary before a bacterium can start to grow, even if there is no optimum, and it is probable that such minima vary from one species of bacteria to another. Evidence in support of such a view is found in H. D. Wright's (1929) observation that reduction restores the power of promoting growth of *Pneumococcus* to media which have lost it through heating. And Dubos (1929[1 & 2]) has emphasized the need of the development of a certain level of oxidation-reduction potential in media before they can grow such bacteria as *Pneumococcus* and *Streptococcus*.

This oxidation-reduction potential work suggests many fresh points of view, e.g. it is clear in view of Cannan's work (1926) that it is not

necessary to assume the action of an oxidase to explain the blueing on exposure to air of the juices of the plant *Mercurialis perennis*. The plant contains a pigment capable of reversible oxidation and present in equal parts of coloured and leuco forms when the oxidation-reduction potential corresponds to rH 12. It is obvious that an rH lower than 12 is maintained in the plant juices since the pigment is present in the plant in a state of complete or almost complete reduction. This rH rises rapidly as soon as oxygen has access to the expressed plant juices, in the same way as it does in nutrient broth shaken in air (Dubos, 1929[1]), and a slight rise of rH is all that is required to promote the appearance of the blue colour.

The observations of the writer with Gordon and Ellingworth (Gordon and McLeod, 1928; Ellingworth, McLeod and Gordon, 1929) show that certain *p*-phenylenediamines are oxidized by bacteria in two stages—the development of a coloured compound, followed by that of blackish pigment. The first stage depends on an accentuation of the spontaneous oxidation of solutions of these compounds exposed to the air, but the second can only be produced by powerful oxidants. The first change probably comes within the range of rH changes which might be evoked by the bacterial ' juices ' exposed to oxygen on the surface of the bacteria, although within the body of the bacteria the coloured compounds appear to be reduced. The second stage involves some more powerful oxidizing mechanism and may be determined by the extent to which iron compounds are incorporated in the cell membrane and function as catalysts in the sense emphasized by Warburg (1914, 1923). The bacteria exerting the latter effect are a more restricted group and are all, according to Nishibe (1926), ferrophil.

Scheme of Types of Bacterial Respiration.

Class 1. Bacteria deficient in catalase, relatively insensitive to KCN and devoid of oxidizing powers for substituted phenols.

Group (*a*) Anaerobes—characters are high activities in reductions dependent on mobilization of hydrogen and feeble reduction of nitrates, development of very low rH values, production of minute traces of peroxide and marked sensitiveness to peroxide. Examples : *B. tetani*, *B. botulinus*.

Group (*b*) Microaerophilic—characters are reducing activities similar to group (*a*), but activation of hydrogen less marked—development of moderately low rH values, production of distinct traces of peroxide. Examples : *Pneumococcus*, *B. acidophilus*.

Group (*c*) Tolerant of oxygen—moderate reducing activity by hydrogen activation, and slight or negligible reducing action on nitrates, and no production of peroxide. Examples : *B. dysenteriæ* (Shiga) and *S. fæcalis* (some strains).

Class 2. Bacteria provided with catalase, sensitive to KCN and incapable of accumulating in their cultures traces of peroxide recognizable by the usual tests.

(*a*) Facultative aerobes or anaerobes—characters are considerable uptake of oxygen, reducing activity both for dyes sensitive to active hydrogen, and for nitrates, development of low rH values in culture. Examples: *Staphylococcus** and *B. coli*.

(*b*) Aerobic growth much more abundant—general characters, rapid oxidation of paraphenylenediamines.

(1) Catalase slight—sensitiveness to H_2O_2 marked—active in reducing nitrates. Examples: Vibrios.

(2) Catalase ++. Examples: *Meningococcus*, *B. pyocyaneus*.

Any scheme of classification must be tentative, and it is not unlikely that farther subdivision may be necessary, e.g. that the respiratory mechanisms of *Staphylococcus* and *B. coli* are distinct.

The micro-organism that is most difficult to place in this scheme is *B. dysenteriæ* (Shiga), for although its sensitiveness to KCN would bring it into the second class its lack of catalase associates it with the first. The failure of this bacterium to produce H_2O_2 is rather puzzling. It may possibly be explained along the lines of rH if it is the case that oxygen only forms H_2O_2 in the presence of a reducing system of a certain level of intensity. The electric potential set up in cultures of the Shiga bacillus has not, so far as is known to the writer, been determined, but the slight reducing activities of the bacillus described above suggest a rather high rH figure.

It is hoped that this chapter has at least suggested the wide and promising field of investigation presented by the phenomena of bacterial respiration.

* The reducing potentials for this bacterium have not been exactly determined so far as I know.

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CHAPTER VIII. THE NOMENCLATURE AND CLASSIFICATION OF MICRO-ORGANISMS.

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Solomon, we are told, knew the names of all the Spirits, and, having their names, he held them subject to his will. The parable may be applied to bacteriology, as to many other sciences. It is not without reason that the International Code of Botanical Nomenclature begins with these words: 'Natural history can make no progress without a regular system of nomenclature which is recognized and used by the great majority of naturalists in all countries'.

Classification is of no less moment than nomenclature, for it should express concisely our ideas as to the relations of natural objects one to another, and sum up the results of our studies, as it were in shorthand. Nomenclature and classification are thus matters of much importance to the bacteriologist, and in the present chapter it is proposed to discuss the general principles which underlie them. More than this will not be attempted, for detailed classification is not a subject for any one writer, not even for debate by the bacteriologists of any one country, but for an international agreement which does not yet officially exist, though we may hope for it in the near future.

Our knowledge of micro-organisms still presents gaps so large that any classification must be incomplete for many years to come. The only bacteria, for example, about which we possess sufficient knowledge for such a purpose are the ones of direct practical importance to mankind—those, namely, which cause disease in man or animals and those which are of industrial value. Yet such properties are merely accidental; the pathogenic bacteria are chance members of different genera which have adapted themselves to a parasitic life; industrial bacteria are kinds which produce in their environment chemical changes which happen to be advantageous to man. But of the vastly greater number of micro-organisms of no apparent practical interest to us our knowledge is almost negligible, although these are the forms from which the better-known ones have sprung and to which they are still closely related. Till these have been adequately studied we have not the material for classifying the bacteria as a whole. Nevertheless, we can name and classify to a considerable extent, and it is the purpose of this essay to discuss the value of the criteria to be employed.

Historical.

To us at the present day it seems so reasonable and natural a thing to name and classify plants and animals that it is surprising to find how late in the history of human thought the practice arose. Aristotle, it is true, endeavoured to classify animals, but after him no one seems to have felt much interest in the matter until the eighteenth century. The very words ' classify ' and ' classification ' date only from this period and, although the first germ of a rational grouping of plants is to be found in John Ray's ' Historia Plantarum ' (1686–1704), it is to the ' Systema Naturæ ' of Linnæus (1735–7) that we must turn for the earliest serious attempt at classification, and it is essentially to Linnæus that we owe the binominal system of scientific nomenclature. The words ' genus ' and ' species ' were terms of mediæval logic, and it is now for the first time that they were adapted to the grouping of natural objects.

For nearly two centuries successive generations of systematic naturalists have laboured to improve the classification of animals and plants, and, although finality has not been reached, it may fairly be said that the principles of naming and grouping the higher members of both kingdoms have been placed upon a satisfactory basis. We shall have to consider how far these principles can be employed amongst the more lowly organized microbes, and it is reasonable, as a prelude, to review the principles and conventions which have been found of service higher in the organic scale.

Nomenclature.

The Latin name of a plant or animal already involves the beginnings of taxonomy for it implies the grouping of species into genera. In addition to the scientific name, which is a Latin one for international reasons, there are for most living creatures, trivial or colloquial names peculiar to each language. This has become so even in bacteriology, and we are in the habit of speaking of the ' diphtheria bacillus ' and the ' tubercle bacillus '. Such trivial names may even have a classical facies and derivation (e.g. ' pneumococcus ', ' gonococcus '), but it is important to remember that these are not scientific names.

For Latin names the binominal rule is now generally accepted, at least by botanists. The use of multinominal names has arisen from the attempt to compress a description into what is really nothing but a tag : such names are now held invalid.

Botanists have bound themselves, by international agreement, to certain conventional rules as regards the scientific naming of plants, and zoologists have adopted a similar course. The botanical code at present in force is that revised by the Brussels International Botanical Congress of 1910 ; this does not explicitly include bacteriology, which it was proposed to deal with later. No one is compelled to abide by the rules

laid down, but every botanist does so because the conventions are the fruit of more than a century of experience, and have been so designed as to secure fixity of names and to avoid all possible ambiguity. The code, which consists partly of fixed general principles and partly of mere recommendations, will be found *in extenso* in R. E. Buchanan's ' General Systematic Bacteriology ' (Baltimore, 1925), while Lehmann and Neumann, in their ' Atlas u. Grundriss der Bakteriologie ' (Munich, 7th ed., 1927), give an excellent epitome of the rules so far as they apply to bacterial nomenclature.

Reference must be made here to a few of the more important features of the code. The *Rule of Priority* affirms that where more than one name has been employed for a species, the oldest, if properly formed, is the valid one ; names are held to date from effective publication. In a young science the first efforts at the recognition and naming of species are necessarily erratic and confused, so that it is needful to fix some arbitrary date for the beginning of official nomenclature. Botanists can go back to the first edition of Linné's ' Species Plantarum ', 1753, but for bacteriology no such date is yet internationally fixed. Some would begin with Cohn's ' Untersuchungen über Bakterien ' (1870–76), while American bacteriologists favour the year (1885) in which the third edition of Zopf's ' Die Spaltpilze ' was published.

Of the two Latin names borne by a species, the first, or *generic*, name must be a substantive : the second, or *specific* name should be an adjective or the genitive of a substantive : rarely a nominative. The only allowable exception to the binominal rule is where two words are required to express a single idea : thus, ' *B. acidi lactici* ' is not held to infringe the rule. It is the practice to put after a Latin name, the name of the responsible author who first employed it. This is usually, but not necessarily, the discoverer of the organism or the person who first described it.

Changes of name. A properly formed Latin name, once responsibly employed, possesses, as it were, an artificial sanctity, however bad it may be. It must not be rejected or altered merely because it appears disagreeable to the ear, or badly chosen, or because some later worker prefers another name. Generic names are in a somewhat different category from specific ones, for, with increasing knowledge, it is often desirable to split up a large genus into several smaller ones. The code contains rules by which such changes may duly be indicated.

Classification.

Underlying all classifications there are certain fundamental principles which must always be kept in mind. The first is that a classification is *subjective*. It does not pre-exist in nature, but is the work of the human mind. It is our interpretation of what we think we see in nature, and it can only go as far as we think we see ; it represents our knowledge at the moment and thus cannot be more than provisional.

A second principle is that of *utility*. We group the facts of nature for our own purposes, and only so far as it is useful for us to draw distinctions. It is possible, by selecting suitable criteria, to divide and subdivide natural objects with any desired degree of refinement : we cease only when further subdivision appears useless. But at any time a further increase in knowledge may make still finer distinctions of practical value. For many years we were content, in our grouping of the pathogenic cocci to have reached such conceptions as the pneumococcus and the meningococcus ; we gave them names and thought we had reached finality. Then it was discovered that there existed several distinct serological races of each of these organisms, and that the recognition of such races was of practical value in the treatment of the diseases which they caused. At once it became necessary to extend our terminology, not, indeed by calling these races new species, but by some provisional form of lettering or numbering, so that each race might be clearly indicated.

A third general principle which now underlies classification is that of *phylogeny*. It is found, in the study of living organisms, that they present different degrees of likeness and unlikeness. Many are so much alike that no useful distinction can be drawn between them : broadly, we call such a group a '*species*', but we shall have to consider more closely, later in this essay, what meaning can be attached to that term amongst bacteria. Certain species have in common some character or characters not found in other species and thus we arrive at the conception of a '*genus*' ; by further processes of integration we reach '*tribes*', '*families*', '*orders*', and '*classes*'. There was a time when the observation and sorting out of these differing degrees of resemblance was merely an exercise in ingenuity, useful in enabling the naturalist to handle his material clearly and concisely, but not known to have any deeper significance. Darwin has changed all this ; acceptance of the doctrine of descent with modification gives us a reasonable explanation of these likenesses and unlikenesses. It has become clear that a classification of living creatures, as opposed to one of inanimate objects, must rest, for its natural sanction, upon phylogeny. This, then, is what we now mean when we speak of a 'natural' classification, and we can understand why, in framing it, all the characters of an object must be taken into account, and not merely one arbitrary one.

The latter method gives us an 'artificial' classification which, however, need not be devoid of value. Schemes of 'determinative bacteriology' offer dichotomic tables of this nature by the aid of which one may commonly determine the name of an unknown organism. Such tables have merely this limited utilitarian value and make no pretence of any phylogenetic sanction. The well-worn simile in which the course of evolution is likened to a branching tree must be presumed to hold good for micro-organisms as for higher forms of life. In the case of the latter, the 'missing links', formed by those members of the trunk and branches which have become extinct, are often preserved for us in fossil form.

Although fossil bacteria are described, we are not in a position to say whether or not the earliest forms of these lowly creatures have become extinct. The existence of gaps amongst the existing forms of bacteria may, nevertheless, lead us to infer that certain forms have failed to survive.

Criteria for Classification amongst Higher Organisms.

The unit in taxonomy is the ' species '. This is an assemblage of individuals which are so alike that no useful distinction, other than those of sex, age, or life-cycle, can be drawn amongst them, and which can breed together, reproducing their like. Within the limits of a species it is recognized that there exist, not only the small individual differences which may form the basis of natural selection, but also, in many cases, subspecies, races or varieties, between which the differences are not sufficiently marked to justify their being regarded as separate species. But it must be remembered that the term ' species ' is a subjective concept, and that opinions may legitimately differ upon such points as these.

In sorting out degrees of likeness it is important to take into account all the characters of natural objects. Linnæus, in framing his classification of plants, used the characters of the reproductive organs alone, and although these are doubtless of fundamental importance, the classification of Linnæus turned out to be artificial and unnatural. It has long been replaced by a more natural one in which other characters are also taken into account.

In the higher animals and plants, the characters employed are almost entirely morphological—differences in size, shape or colour which can be recognized with the naked eye or under a low magnification. It is almost true to say that, until the facts of bacteriology made it necessary, the idea of employing physiological characters for purposes of classification had never been raised. The exception to this statement lies in the criterion of mutual fertility ; it is a general rule that groups of living creatures far enough apart to be deemed separate species are found to be mutually infertile or at the most to produce sterile offspring. This rule, however, is by no means an absolute one, especially amongst plants.

It is interesting to note, in passing, that this exclusive reliance on morphology is in no way due to the absence of suitable physiological criteria. Now that bacteriologists have devised serological methods for discriminating between different bacteria, similar methods are being found applicable to animal species, amongst which the intensity of precipitin reactions seems proportional to phylogenetic affinity. The facts of blood-grouping indicate the existence, in man himself, of serological races which are probably comparable to those found in the pneumococcus.

Contrary to what might have been expected, it has been found that the most trustworthy characters for indicating natural affinities are not always those that are most conspicuous or of most obvious utility to their possessors. Such characters may be recent acquirements, or a response

to special needs ; real affinities are often better displayed in characters of apparently more trivial nature and of little seeming utility. These considerations have been discussed with great lucidity and wealth of detail in the Chapter on Classification in Darwin's ' Origin of Species '.

The characters employed to separate genera are necessarily of a more fundamental kind than those which distinguish species, for the cleavages between genera are deeper and presumably of greater antiquity. Where possible it is desirable to employ, for generic characters, the structure of the reproductive organs.

Genera differ greatly in size ; some contain few species or even one only, others a large number. It is noteworthy that in small genera the distinctions between the component species are usually well marked, whereas in very large genera specific distinctions are apt to be slight and ill-marked, intermediate varieties being common. A reasonable explanation of these facts would be that the small genera are ancient ones which have already fulfilled their evolutionary destiny, and are no longer actively varying, while the large genera, usually numerous in individuals as well as in species, are those which are at the moment succeeding in the struggle for existence, varying actively and not yet fully differentiated into sharply defined species. The brambles, the roses and the saxifrages are ' dominant genera ' of this kind, and they present great difficulties in classification. It is almost impossible to give a separate name to every form, and the usual practice of botanists is to regard the commonest and most wide-spread forms as species and the intermediate ones as subspecies and varieties.

Difficulties in Classifying Micro-organisms.

Such being the general principles which have been reached by taxonomists in the classification of the higher plants and animals we have now to consider how far we can apply these principles and conventions to bacteria and other micro-organisms. Can we regard the classification of bacteria as merely a downward extension of systematic botany, and that of the animal micro-parasites as a branch of protozoology ? Every protozoologist would reply to the latter question in the affirmative, but the answer of the bacteriologist is not quite so easy. The special difficulties which here arise may be considered under several headings, but it may be stated at the outset that no substantial reasons can be found for refusing to accept the general principles which have been discussed in the foregoing pages.

The Systematic Position of Micro-organisms.

A primary difficulty is that of referring certain groups of microbes to their appropriate kingdom. We are often on the borderland between animals and vegetables, and we are dealing with organisms so minute that their true characters, and especially their physiological properties are sometimes hard to ascertain. Some, such as the Entamœbæ and the

intestinal Flagellates, belong by every criterion to the animal kingdom and fall naturally into place amongst the Protozoa. Others, such as the mould fungi and yeasts are assignable no less clearly to the vegetable kingdom. The earliest observers, with their imperfect means of investigation, had described all the minute creatures they saw as animals ; it was not till the middle of the nineteenth century that first Naegeli and then Cohn proclaimed the vegetable nature of bacteria. So gradual is the transition from the mould-fungi, through the streptothrix group and the acid-fast bacteria, to ordinary bacteria, that there are few who do not agree with Naegeli. As further evidence, it may be added on the morphological side that bacteria present a separable cell-wall, and, on the physiological side that many, at least, exhibit the characteristically vegetable power of synthesizing protein from inorganic sources.

There remain, nevertheless, certain forms, closely allied to the bacteria, which are very difficult to place—notably the Spirochætes. To revive Haeckel's third kingdom of ' Protista ' for organisms so low down in the scale that they cannot definitely be assigned to either of the other kingdoms, may be a useful expedient, but is a doubtful gain, for it necessitates two arbitrary lines of demarcation in place of one. It is to be hoped that, in the fulness of time, the fundamental biological properties of such organisms will be so far elucidated that they will be found to fall into one or the other of the usually accepted kingdoms. Meanwhile judgment must be suspended, as it must, for more obvious reasons, in the case of the invisible viruses or so-called ' filter-passing ' organisms. Here our ignorance is almost complete : they are possibly a heterogeneous group, but in the case of creatures which we cannot see and whose very existence is in some instances a matter of inference only, it is idle to talk of classification in its usual sense.

The Existence of Species amongst Bacteria.

In order to understand the controversies which raged, in the early days of bacteriology, between the exponents of ' pleomorphism ' and their antagonists, we must not forget the disadvantages under which the early workers laboured. Until Koch had introduced solid culture-media and shown how pure growths could be ensured by their use, contaminated cultures were probably the rule. Microscopes, too, had not attained their present perfection—a serious matter at a time when morphological criteria were alone available. We cannot wonder that men such as Hallier and Naegeli fell into grave errors. In its extreme form, as taught by Hallier, the doctrine of pleomorphism held that all bacteria belonged to a few infinitely variable species. Naegeli, a few years later, did not dispute the possible existence of separate species amongst them, but failed to find adequate characters for separating them, and, indeed, despaired of attaining pure cultures.

The man whose labours first dispelled these errors was Ferdinand Cohn, who, in the ' Beiträge zur Biologie der Pflanzen ' (1872 and 1876),

declared his belief that definite genera and species existed amongst bacteria, though he was partially baffled in his attempts at exact classification, for the times were not ripe. Cohn's work was truly a landmark in bacteriological taxonomy, for he was the first to insist upon the value of other than morphological characters. He was prepared to take into account pigment production, fermentations and pathogenic power in distinguishing between allied forms. Robert Koch established Cohn's views upon a still firmer basis; his methods of separating bacteria and obtaining pure cultures enabled him to affirm that each transmissible disease was associated with its own specific microbe. Henceforth the doctrine of specificity became firmly entrenched, and this, in Neufeld's words, may be regarded as ' the quintessence of Koch's life-work '.

During the last 30 or 40 years the characters of the more important pathogenic bacteria have been studied with an ever-increasing minuteness of detail, and with the confidence which modern technique gives in the purity of cultures; we can, if we choose, deal with a culture known to have been derived from a single cell. We have acquired a reliable knowledge of the range of variation possible within the limits of a single species, and, in consequence, we are in a far better position than Koch to judge of the existence and permanence of specific distinctions amongst bacteria. Nevertheless, not a few workers have assailed the doctrine of specificity in its absolute form. Bacteriology differs from most of the so-called ' observational ' sciences in that it is to a high degree an experimental science too. Experiment is fruitfully employed in all branches of biology, but the readiness with which bacteria can be cultivated artificially, the rapidity with which one generation succeeds another and the ease with which variations can be introduced into the environment render the experimental method of peculiar value to the bacteriologist. At different times writers have claimed to have effected the transformation of one bacterial species into another by artificial means—not, be it understood, into an entirely remote species, but into an allied form commonly regarded as distinct. It has been asserted, for example, that virulent diphtheria bacilli have been changed into such harmless congeners as *C. xerosis* or *C. hofmanni*, and that hæmolytic streptococci may change into the relatively harmless and non-hæmolytic *Streptococcus viridans*. It is significant that the method by which such changes have been brought about has frequently been animal passage—a method fraught with possibilities of error. Somewhat similar changes, however, are claimed to have been produced in the test-tube in cultures derived from a single cell. It is well established that certain properties, such as virulence or hæmolytic power, may be modified or lost when a microbe is grown under unnatural conditions, and we should be cautious in accepting changes of this sort as evidence of permanent transformation of one bacterial species into another. It must, indeed, be said that, while modern experimental work has revealed a latitude of variation within the species which was unsuspected by earlier workers, it has shown that such variations are

largely degenerative in type and due to artificial conditions. It has not seriously weakened general belief in the validity of the ' species concept ' amongst bacteria in the same sense as amongst higher plants.

But just as amongst higher plants there are dominant genera rich in somewhat ill-defined species and with many transitional forms, so amongst bacteria we find analogous groups of successful and actively varying forms which present grave difficulties to the systematist. For obvious reasons these are the very forms which most frequently obtrude themselves upon the worker, and hence there may arise an erroneous impression of lack of fixity in bacterial characters. The normal flora of the alimentary canal offers more than one example of such dominant genera, but one only must suffice.

During the past twenty years a great deal of attention has been paid to the classification of the Streptococci. No approach to finality has been reached, and it would seem that we have here a genus eminently successful in the struggle for existence, actively varying and constituting a group of ' species in the making ' which it is best consciously to approach as such. By statistical treatment of the characters of a very large number of individual strains one may determine their relative numerical frequency. The most abundant may be regarded as ' centres of variation ' around which the intermediate forms are grouped, and these centres may be treated as nascent species to which we may conveniently give names. In genera such as these, this more fluid concept of the term ' species ' probably corresponds better with the facts of Nature than the more rigid one which serves well enough in less actively varying genera. It is no reproach to the bacteriologist that he cannot discover sharp limits to species when, in fact, none such exist.

Absence of Permanent ' Types '.

When a naturalist describes a new plant or a new insect, the specimen from which the description was made is nowadays carefully preserved in some museum or other collection as the ' type ' of that species. Anyone who later wishes to ascertain whether a specimen under his hand belongs to that species can go and compare it with the type. It is a misfortune that there is an impermanence about bacteria which practically forbids the employment of this invaluable mode of record. It is possible, no doubt, to carry on subcultures of an organism for a great many years, but, even apart from the dangers of accidental loss or of contamination, all living organisms tend to undergo slow degenerative changes under prolonged artificial culture, and there is no guarantee that the properties found after a term of years will coincide with those of the freshly isolated organism ; there is, indeed, a strong presumption that they will not.

More hopeful are methods which preserve the organism in a state of suspended animation. In the case of spore-bearing microbes, the dried spores may be preserved with care for at least ten or twenty years. Hermetically sealed agar stab cultures of many non-sporing bacteria will often be

found alive after five or ten years. Better still is the method, devised by Homer Swift, of drying at very low temperatures, by which even delicate organisms such as the hæmolytic streptococci may be preserved for many years, not only alive but with no loss of virulence. But even with the best of these methods, subculture must be resorted to, even though at long intervals, and slow changes probably occur.

In a large proportion of the described species of bacteria there has been no attempt to hand down living examples to posterity. The museum of the bacteriologist is for the most part a museum of labels from which the specimens themselves have long disappeared. Moreover, the labels are often so inadequate that we are left in doubt as to their meaning. The older workers were necessarily in ignorance of many of the criteria upon which we now lay the greatest stress, and it follows that the precise identification of some of our commonest organisms (e.g. *B. coli communis*) has been a matter of guess-work, though now agreed upon by general convention.

It need hardly be said, in view of the foregoing remarks, that the characters by which a species is defined must be those presented on its first isolation from its natural habitat, and not those which it may come to present after prolonged artificial culture.

Criteria for Classification amongst Bacteria.

Having now considered in general terms the question of specific distinctions amongst bacteria, we must discuss in some detail what are the actual characters available for their classification, what are the relative values of these and what limitations must be placed upon their use. These things form the crux of the whole subject.

In a discussion of this sort it must be kept in mind that there is one important difference between the characters available amongst bacteria and those of higher plants and animals. In the latter we are accustomed to draw our characters from study of the individual. Whilst we can do this, up to a point, with a bacterium, most of the characters which we study in these minute creatures are derived from observing their behaviour in the mass; there seems no possibility of studying the physiological properties of the individual. Now, granting purity of culture, granting even a growth derived from a single cell, this mode of study assumes the physiological equivalence of all the individuals in the massed growth. It is probable that in most instances no fallacy is introduced by such an assumption, but it is also probable that the assumption is never absolutely true. In the case of a pathogenic organism it seems almost certain that different individuals in a culture differ in virulence: the effect of animal passage in enhancing virulence is presumably due to a weeding out of the inefficient while the competent are given a chance to show their worth. Similar variations probably exist with regard to other properties. In the case of certain species more striking differences are found—for instance,

the specific and group forms of some Salmonellas, while pneumococci and hæmolytic streptococci are liable to give two forms of colony which differ in virulence and in serological properties. The importance of such facts lies in this—that two men, starting from the same culture, may come to have in hand subcultures with different properties ; but if the dangers are known the risk of fallacy becomes much less.

Morphology.

Inasmuch as outward form has been found all-sufficing in higher plants, it naturally takes precedence over other criteria amongst micro-organisms. The first workers in bacteriology approached the subject with no idea of any other means of discrimination, and when, for reasons previously mentioned, they found themselves involved in hopeless confusion, they took refuge in the doctrine of pleomorphism. Now that the experience of fifty years has taught us the limits of variability and shown us how largely it depends on external circumstances the fact of pleomorphism is viewed with unconcern ; where we are in doubt we turn to other specific criteria. Taking morphology in a wide sense, to include not only size, shape and planes of division, but the presence, number and distribution of flagella, the capacity of forming resting endospores and even such cytological details as the presence of polar and other granules—using the term, in fact, for everything that we can see with a microscope —we can to-day employ morphological characters with confidence, so far as they will carry us. This is perhaps not very far, but at least morphology gives us our fundamental grouping of the bacteria proper into ' spheres, rods and screws '. There are doubtless occasional difficulties as to the boundaries between these groups. Certain streptococci, when they grow on a favourable solid medium, have a disconcerting way of growing out here and there into rods, but this can deceive only the inexperienced. It may be difficult to distinguish between a very short rod and a slightly oval coccus : there may be doubt as to whether or not an apparent bend in a rod is to be taken as indicating a segment of a screw. But, on the whole, systematic bacteriologists are at one as to the validity of these primary groups.

Morphology can, however, do more for us than this : it can furnish us with features which are of generic value ; indeed, many hold that genera should only be founded on morphological characters.

Size. This is a feature of less value than shape because it is more liable to vary with external circumstances. In any given species, diameter presents greater constancy than length. Rod-shaped bacteria multiply by transverse division in one plane, and the rate at which division occurs is greater the more favourable the conditions for growth. The typhoid bacillus, for example, grown on gelatin at room temperature, exhibits much longer forms than when grown on agar at 37° C. In any case, averages of many individual measurements are requisite, if the figures are to have any value.

When we approach the lower limit of size amongst bacteria we become involved in certain difficulties. The lower limit of visibility with the best present-day microscopes may be put at $0{\cdot}1$ to $0{\cdot}2\mu$, and it was at one time thought that no bacteria were less than $0{\cdot}3\mu$ in diameter. But there is some evidence that certain bacteria, at a given phase in their life-cycle, may pass into an invisible stage capable of passing standard filters supposed to keep back bacteria. The relation of *B. pneumosintes* to Pfeiffer's bacillus may possibly be of this nature, but the whole question of a filter-passing stage in the life-cycle of ordinary bacteria is at present quite unsettled.

Shape. Amongst the spherical organisms shape can tell us little, though even here the flattened surfaces of the apposed faces of the pairs in certain Gram-negative cocci is characteristic. But another factor comes in to assist the morphologist amongst the cocci, namely, the sequence of the planes in which division occurs. There may be one plane only, as amongst the rod-shaped bacteria, in which case the resultant grouping is a chain (Streptococcus), or there may be two alternating planes at right angles to one another (Merismopedia) or three planes at right angles in regular sequence, yielding cubical packets (Sarcina). Or again there may be no systematic order in the planes of division, so that irregular groupings result (Staphylococcus). Features such as these seem to be of generic value.

There are some rod-shaped bacteria which present characteristic shapes ; the definite clubbing of the diphtheria bacillus and its allies is a case in point. Some bacilli have square-cut ends, others are pointed. Such features, when constant, may usually be regarded as of generic significance. The presence or absence of a capsule is probably of less value as a criterion because its development varies with circumstances even in the same species : amongst pathogenic bacteria it is chiefly restricted to individuals growing within the animal body, and is not usually seen in artificial cultures.

Flagella. It can hardly be doubted that the definite presence of flagella and of motility should rank as a morphological feature of high taxonomic worth. If, for example, we contrast the dysentery bacilli with the organisms of the enteric group, the absence of flagella in the former and their presence in the latter must be deemed a generic character. Again, the disposition of the flagella, when present (monotrichous, lophotrichous or peritrichous—to use Fischer's terminology) should equally be of generic value. It is true that instances can be quoted in which one of two closely allied organisms is motile and the other apparently not ; the typhoid bacillus, for example, is flagellate, while the closely allied *B. pullorum* seems destitute of motility ; the glanders bacillus has always been regarded as non-motile, whereas the very nearly allied *B. whitmori* is motile. Before, however, laying too much stress on such discrepancies, it must be remembered that there are two circumstances which may render the application of this criterion difficult. The first is

that the demonstration of flagella and of motility may be, in some cases, a matter of great difficulty even to the skilled worker (e.g. in *B. tuberculosis rodentium*). The second is that these characters are readily lost on artificial culture, if the environment is at all unsuitable (H- and O-forms of *B. proteus*). The criterion is one, therefore, to be employed with due caution ; the absence of flagella must only be presumed after an organism has long been studied by many observers under optimum conditions.

The formation of *resting endospores* is a less uncertain criterion, though it may be needful to know the exact physiological requirements of a species before assuming that it forms no spores. In those species which produce them, the average measurements, shape and position of the spores are reasonably constant features.

Acid-fastness and the reaction with *Gram's stain* may almost rank with morphology in taxonomic value.

The delimitation of the main groups of bacteria and in many cases of their genera is, however, as far as morphology can take us ; when we pass to the several species comprised within a genus, it breaks down as a guide. It is rarely, indeed, that an experienced bacteriologist will be found ready to affirm with certainty that an organism belongs to a given species merely by looking at it with a microscope.

It has been the task of the past fifty years of bacteriology to study the more important micro-organisms in every way, step by step as the existence of other than morphological characters become revealed and as the technique for their investigation became perfected. And it may here be remarked that, whereas morphology is essentially concerned with the individual organism, other characters can be studied only in the massed culture.

Cultural Characters.

No sooner had Koch devised solid culture media, on which the progeny of a single bacterium form a discrete colony, visible to the naked eye, than it became apparent that the colonies of different species, and their massed growth in pure subculture, differed visibly in physical characters—in size, shape, consistency and colour. A new set of useful diagnostic criteria was thus revealed.

Such characters, however, must be employed with a due sense of the possible fallacies which attend them. Small changes in the composition or reaction of a culture medium may bring about appreciable differences in the characters of bacterial colonies, and it follows that, when two species are being compared, they should be grown side by side on the same medium or in tubes from the same batch.

Again, within the limits of a single species, there may occur variations, spontaneous or induced, greatly influencing the appearance of the colonies. The H- and O-forms of the Proteus bacilli offer an extreme instance, and others are furnished by the ' rough ' and ' smooth ' variants first described by Arkwright and now known to occur in many bacterial genera. In these

variants, not only do the colonies on solid media differ in aspect, but the appearances in broth culture may be quite unlike, the smooth forms showing uniform turbidity and the rough ones a deposit with clear supernatant fluid. The formation of pigment is another variable ; it may need optimum conditions for its full development (*Staphylococcus aureus*, *B. prodigiosus*), and these must be known for each particular case. Moreover, varieties with no pigment sometimes occur in a naturally pigmented species.

Temperature Requirements.

In this respect we can divide bacteria into three, or perhaps four groups. (1) The great majority of the organisms found ' wild ' in Nature grow best at the ordinary summer temperature of the air. (2) Those which have adapted themselves to a saprophytic or parasitic life in relation with warm-blooded animals have an optimum temperature round about 37 or 38° C. (3) The thermophilic bacteria of hot springs or fermenting manure heaps flourish at temperatures fatal to most micro-organisms. (4) The as yet little-known moulds and allied organisms found in cold-storage plants are able to grow at a temperature not far removed from 0° C.

Characters such as these are of importance because they indicate evolutionary adaptations, but they are hardly of practical value as criteria between one species and its neighbours. It is a noteworthy fact, however, that any given species practically always falls into one or another of the groups mentioned.

Oxygen Requirements.

The subject of respiration amongst bacteria is one which has only recently attracted attention on its chemical side, but the main facts of ' aerobiosis ' and ' anaerobiosis ' have long been recognized. More recently a ' micro-aerophilic ' group has been established. These matters are discussed elsewhere, and here it need only be remarked that, in respect to classification, oxygen requirements and mode of respiration stand on much the same footing as temperature needs—of broad general interest, but rarely, if ever, of value as specific criteria. The strictly anaerobic, spore-forming bacilli do, however, fall together as a natural genus of which the mode of respiration is a constant character.

Fermentative Powers.

Although bacteria and some other fungi are regarded as being themselves ' living ferments ', there is no reason for doubting that they effect chemical changes in their environment by the production of enzymes similar to, and perhaps identical with, those with which we are familiar in higher plants and animals. Of the forms of chemical change brought about by bacterial action, there are two which have been in use for many years as a means of discriminating between different species. One is the attack upon proteins and the other the breaking down of carbohydrates, alcohols and allied food-stuffs.

Two familiar characters dependent on *proteolysis* are liquefaction of gelatin and putrefaction. The liquefaction of gelatin has been used as a specific criterion ever since Koch introduced gelatin media. When, as is commonly the case, the liquefaction is pronounced, the criterion is a good one, but some bacteria produce the change so slowly and so feebly that it is difficult to say whether the result of the test is positive or negative. The more advanced proteolytic changes comprehended under the term putrefaction are almost always the work of anaerobic bacteria. When the chemistry of the resulting products has been fully investigated it will probably be found that different species vary in the end-products they yield. There are, moreover, aerobic bacteria which, while not causing the stinking changes of actual putrefaction, nevertheless give rise to certain products of protein cleavage, such, for instance, as indole, which may be made the basis of useful specific tests.

The *saccharolytic powers* of bacteria are amongst the most widely used of specific criteria. The range of fermentable sugars and alcohols is extensive and it is possible to set a bacterium tasks of differing degrees of severity, since some sugars, such as glucose, break asunder with ease, while the molecules of others are less susceptible to attack. The usual test which is employed to ascertain whether or not fermentation has occurred is the formation of acid, as revealed by some indicator in the medium, accompanied or not, as the case may be, by the evolution of gas. This is a rough method and needs some consideration. In the first place the acid formed may have some other source than the sugar presented. In the second place it is only when the attack on the sugar is a vigorous one that it is thus manifested ; slighter degrees of change may only be revealed by careful quantitative chemical analysis, and, in fact, it has been shown chemically that lactose may be broken down to a slight extent by a microbe which commonly passes as a non-lactose-fermenter. The question is thus a quantitative one, though the test is none the less practically useful on that account. This being so, we can better understand some of the anomalies which are sometimes encountered, such as apparent temporary loss of a fermenting power previously possessed, or gain of the requisite power when an organism is ' trained ' by habitual presence of a sugar. A quantitative change is more intelligible than the gain of an entirely new property.

Similar considerations apply to the occurrence of visible gas formation. Gas must be formed whenever a sugar is broken up, but in small quantities it passes into solution or escapes into the atmosphere ; only when fermentation is vigorous does it collect in visible amount. It is not uncommon to meet with varieties of gas-forming bacteria which produce no visible gas, and this may be presumed to be a mere quantitative defect.

The chemical changes in a bacterial fermentation seem constant under uniform conditions. Analysis of the end-products and of the gases evolved may be of service in difficult cases. This is a more

refined and reliable method than the rough criteria of acidity and gas production, but is hardly adapted for routine use.

The fermentations and other changes which have just been mentioned by no means exhaust the list of chemical tests available for distinguishing amongst bacteria, but they are sufficient to indicate their value. Too much stress must not be laid upon such tests, because, especially under artificial culture, changes may occur in the list of sugars apparently attacked by a given bacterium. Such changes seem commoner with some sugars than with others, so that all are not of equal value as test substances. But on the whole, if due allowance be made for occasional lapses of this kind, chemical changes in the medium of growth afford some of the most useful of specific criteria.

Antigenic Characters.

The power possessed by the bacterial body, and by certain of its products, of calling forth, in the blood of living animals, substances or properties of an antagonistic nature, is unquestionably the most delicate of the means available for determining the identity or non-identity of two bacteria. It is indeed too delicate for the mere naturalist, though not so for the immunologist. The formation and properties of antibodies are fully dealt with elsewhere in this work. Here it must be noted that each separate protein (or, it may be, protein-lipoid complex, or other compound capable of acting as an antigen) gives rise to its own special antibody, and that the reaction between antibody and antigen is of a highly specific character. Study of the antibodies evoked in response to the injection of bacteria into the animal body—precipitins and agglutinins being those which have yielded the most information—indicates that the bacterial substance consists of a number of different ingredients, some common to a number of different kinds of bacteria, others peculiar to the one kind—some readily destroyed by heating, others capable of resisting actual boiling. This conception of bacterial structure, sometimes called the ' mosaic ' theory, has not been without its critics. It must not be taken literally and crudely as suggesting different proteins disposed in lumps about the cell ; we do not know the disposition of the various ingredients of the bacterial body, but there can be no question as to the existence of multiple ingredients. Chemistry, indeed, is already beginning to come to the aid of the immunologist, as, for example, by the extraction of different compounds from different serological races of the pneumococcus. Nevertheless the possession of a given substance in common by two bacteria is no absolute proof of intimate phylogenetic relationship, for it has been found to occur, in some instances, in two organisms widely separated in classification (a particular strain of the pneumococcus, and a race of Friedländer's bacillus).

As regards agglutination it seems clear that the antigens and their corresponding antibodies fall into two main groups. In species possessing flagella, the chemical composition of these structures must be different

from that of the main body-substance; we can distinguish between 'flagellar' antigens, which are thermo-labile and 'somatic' antigens which resist heating. There are corresponding agglutinins and the appearances differ in the two types of reaction. By such means and with the aid of the absorption technique, it seems possible to effect a sort of provisional analysis of the antigens present in any given organism. Such an analysis has been attempted on an elaborate scale by Bruce White for the genus Salmonella and its near allies, and he has gone so far as to reconstruct a phylogeny of the group on this basis. Now that we have become aware that chemically identical antigens may occur in bacteria which are certainly not closely related, we can perceive the danger of drawing too sweeping conclusions from such facts, though it is reasonable to allow them a good deal of weight.

The high degree of specificity and delicacy of these immunological reactions constitutes at once their strength and their weakness for purposes of classification. To the experienced worker, familiar with the pitfalls which may attend their use, such reactions constitute a final court of appeal as to the precise identity of two organisms. To the inexperienced they may at times prove a source of error. There are the facts of diphasic variation seen in many Salmonellas, there are the various degenerative changes occurring in long cultivated strains ('rough' variants), there are the known instances in which an identical antigen occurs in different species—all these may lead the unwary astray. In any event it would seem that immunological reactions tend to go beyond the limits which attach to 'species' in the natural history sense. In many cases we can split up a species into a number of serological races, the conception of the species being based, in such instances, on the sum of other criteria. Sometimes the existence of serological races may be of apparently small consequence, for the races, deemed separate on grounds of agglutination, form one common toxin: this seems to be the case with the diphtheria bacillus, the tetanus bacillus and some of the organisms concerned in gas gangrene—all, it may be noted, species forming a soluble toxin. But there are other species (meningococcus, pneumococcus) in which serological races have to be taken very seriously into account in efforts at vaccine, or serum, treatment.

It has been hinted above that actual chemical differences have been shown to exist between the serological races of a given organism. It is not impossible that, with the progress of biochemistry, chemical differences may come to play a part of importance in the classification of bacteria. Such a likelihood is, indeed, already foreshadowed by the work of Mrs. Lancefield in America, which indicates amongst streptococci and their congeners (including pneumococci), (1) a nucleoprotein common to all species, forming a necessary basis for all the antigenic substances which they may yield; (2) species-specific substances, polysaccharide in nature, peculiar to each species; and (3) type-specific substances, again nitrogenous in nature, peculiar to individual serological races.

Pathogenesis.

It might be thought, at first sight, that the power of producing disease was a character of high importance in classification. Up to a point this is so, but there are circumstances which forbid our placing it in the first rank. The production of disease does not rest solely with the microbe : the ' disease ' is a reaction on the part of the host, and a character which depends upon the variable resistance of an external factor is not a very satisfactory one. Moreover, the intrinsic virulence of a given microbe is subject to wide fluctuations. When conditions are favourable, as at the height of an epidemic amongst a crowded susceptible population, the virulence of the pathogenic agent may be greatly enhanced. Under unfavourable conditions, such as those of artificial cultivation, virulence is apt to wane and pathogenic power ultimately to become extinct. There seem, too, to be species which present, even in Nature, virulent and non-virulent varieties : thus, the diphtheria bacillus, as encountered in the fauces of a mixed school population, is found in these two varieties, and there seems no constant difference between the two in any other respect.

Virulence may be regarded as a late acquirement in the evolution of a species and still a labile one. Nevertheless, although pathogenic organisms may present non-pathogenic variants, it must be conceded that definite taxonomic value must be assigned to pathogenic power, even if this is only occasionally present in a species.

General Conclusions.

Looking back upon the various criteria which have been discussed in the preceding pages, it would seem that they are adequate for discriminating between bacterial species in the majority of cases. In any given case it is the sum of the various characters which counts. Where, as in certain dominant genera, specific distinctions are somewhat elusive, we must fall back on the notion of ' centres of variation ' as established by statistical study of a number of characters.

Few bacteriologists would deny that bacterial species fall naturally into groups of the value of genera. These groups tend to cluster around some characteristic species, as for instance the members of the genus Mycobacterium around the tubercle bacillus, or Corynebacterium around the diphtheria bacillus. Such a representative species forms the ' genotype ' of the genus, and nowadays, when a writer describes a new genus, he deliberately associates it with a particular species, as genotype. This practice hands down unambiguously to posterity the precise idea which the author had in mind in creating the genus.

Modern Attempts at Classification.

The foundation of all later classifications of Bacteria was laid by Ferdinard Cohn. As knowledge improved, succeeding writers, amongst whom may specially be mentioned Zopf and Migula, effected corresponding improvements in grouping. An important advance lay in the stress put by Migula on the presence, number and distribution of flagella.

If Cohn's work formed the first great landmark in bacterial classification, the second may perhaps be assigned to the publication of the first edition of Lehmann and Neumann's ' Atlas und Grundriss der Bakteriologie ' in 1896. Here, for the first time, we find clearly laid down the principles and conventions governing nomenclature applied to Bacteriology, and a definite application of them to the subject at issue. We owe to these authors the establishment of the genera ' *Corynebacterium* ' and ' *Mycobacterium* ' and their affiliation with the Actinomycetes.

It is curious that British and French bacteriologists, who have shown great activity in other fields of their science, should have displayed almost complete indifference to systematic bacteriology. Up to the earlier years of this century the chief efforts at taxonomy were, as we have seen, due to the Germans : in more recent years the mantle seems to have fallen upon the United States, where the Americans have been greatly exercised over bacterial nomenclature and classification. In September 1917 appeared a preliminary report on ' Characterization and Classification of Bacterial Types ' by a Committee of five persons appointed by the Society of American Bacteriologists. This was followed in May 1920 by a final report in which what has now become known as the ' American Classification ' was put forward. This is the most recent attempt at improved grouping, and it is only fair to say that it is a sincere and painstaking effort to bring order into a subject which, with the great increase in knowledge of the past twenty years, had again become much confused. Nevertheless it is, as it were, only a local effort, as yet without the sanction of international agreement behind it ; indeed it is not universally accepted even in America. One of its chief features lies in the creation of a number of new genera to indicate the natural groups into which the rod-shaped bacteria fall, and new generic names have been formed, on both sides of the Atlantic, from the names of prominent workers in the science. Sometimes the result has been not inartistic ; such names as Salmonella, Brucella and Pasteurella are pleasing to the ear and pay a well-deserved compliment to distinguished men. Nevertheless, marriage between Northern names and Latin terminations is seldom a happy practice, and we might well have been spared such barbarisms as ' Escherichia ' and ' McIntoshiella '.

In this essay the principles of classification have been illustrated almost entirely from the bacteriological side. This has been intentional, for bacteria present at once great difficulties in classification and a great wealth of ascertained fact by which we may hope to overcome those difficulties. There is, however, no reason for doubting that the principles which have been discussed apply equally to other branches of microbic life. Wherever, indeed, in these other branches the life-cycle is more complex than amongst bacteria, the characters available for differentiation become to that extent more numerous and the task of the taxonomist the less hard.

CHAPTER IX. VARIATION.

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Introduction.

General Considerations. Stability and Variability.

In the early days of bacteriology the varied forms of micro-organisms in cultures, observed microscopically, led to the view, held by Nägeli, Buchner and Wernich, that these forms of life were extremely variable, especially under changed external conditions. They believed in very rapid and almost unlimited changes in the characters of the individuals of a single species. Nägeli (1877) maintained that each species of bacteria could occasion the formation of lactic acid, putrefaction and various forms of disease. This view was later modified by Buchner to the extent that he considered each species of bacteria to be distinct in spite of being extraordinarily variable. The variability of morphology and difficulty in ensuring purity of culture even suggested that bacteria might be a stage in the life-history of higher fungi. Koch (1881) and Hueppe (1883) showed that many of the supposed changes, especially the assumption of a proteolytic function as a new character by certain species, were due to impure cultures, which was partly explained by the presence of resistant spores in materials supposed to have been sterilized.

Further, an increasing number of distinctive and very persistent characters was discovered and enabled a more exact definition to be given of many species. Particularly, agglutination and other specific characters related to immunity, and certain fermentative properties, were observed under varied conditions over a long period and were found to be very constant.

Cohn (1875) took the view that the individual species preserved their morphological and other characters with great constancy. This view was strengthened by the work of Koch, whose technique with solid culture media greatly facilitated the isolation and observation of pure cultures. Variety in size and shape often seen in cultures which had been believed to contain only a single species were freely set aside as being usually due to contaminating bacteria or to the original culture having throughout consisted of two or more kinds. It was then found that young cultures obtained from single colonies on solid culture media usually contained bacteria of remarkable uniformity, both as regards the morphology of the colonies and of the individual organisms, and the staining properties of the latter.

It was admitted that the bacteria in old cultures or when viewed fresh from the body of an infected animal presented shapes and staining

properties which were often very varied. This fact was, however, accounted for by the hypothesis that in such cases 'involution', or processes of deterioration tending to death, were taking place and that such altered bacteria were not in a healthy condition. In healthy young cultures, on the other hand, great uniformity and absence of variation was strongly claimed for each species. Koch especially, maintained the fixity and unalterable character of species and even at first doubted that 'attenuation' of virulence was due to other causes than contamination or the death of a proportion of the individual bacilli in a culture.

At this period, and for many years afterwards, this hesitation to admit variation was dominant, and was indeed, it appears, a necessary stage in the advance of bacteriology as a branch of pathology. In claiming the ability to recognize an infecting micro-organism and to recover it from its host, or under other varying circumstances, it was essential to believe that it should present a definite picture, and that cultures of the same microbe from different sources should be of uniform appearance and give uniform characters of growth and of biochemical and other reactions. If such continuity had not been postulated, identification would have been impossible. Though, no doubt, sometimes leading to error, an insistence on uniformity of morphology and other characters enabled the constant relation of certain kinds of bacteria to special habitats, diseases, and to special chemical and physical processes to be firmly established beyond cavil, and the science of bacteriology to be rapidly and surely extended. This early caution was the more necessary since the differential characters which had been studied at that time were few, and consequently the observed differences between species might be abolished by very slight changes in the culture. By the stringent rules laid down, a number of variant forms were unnecessarily discarded or neglected, but the demand for uniform characters under uniform conditions was justified by the regular results and the exactness of the correspondence which could be demonstrated between parasite and animal host in a large number of instances. As the number of recognized noteworthy distinguishing characters increased, it became proportionately common for observations on variability in one or other character to be recorded.

Limits of Species and Range of Variation.

There are wide and varied differences between races and strains of bacteria from different sources, but believed to be of the same species. Since the evidence is not complete that these have originated from one parent strain within recent times, they will not be considered in this chapter, except in the statement that the lines along which they diverge on the whole resemble those of variation which occurs under observation. The variations inside the animal body or in a state of nature may be greater than those observed to take place in the laboratory, and on the other hand, some laboratory variants would not be recognized if isolated from the outside world.

As the record of new natural differences has multiplied, it has been found possible to separate more fixed species or subspecies, but the difficulty in deciding which strains are worthy of specific rank is often as great as among closely allied groups in the higher orders of living things.

Among the earlier records of such variation may be mentioned the absence or presence of a capsule, the cessation of motility, the unusual length or breadth of the individual bacilli and the growth of bacilli or cocci in long chains or threads. Among changes in function the failure or diminution in the production of pigment by staphylococci or *B. prodigiosus* and *B. pyocyaneus*, and the loss or gain of pathogenicity are of common occurrence.

Such observations have been very greatly extended, and variability has been shown to affect many species of bacteria, and in those which have been carefully studied the great majority of described characters, including the serological properties, have proved liable to variation. This statement must not be taken to imply that all the characters of a well-established species, group, or family of bacteria may change simultaneously or in such a way as to make them identical with those of another group of species. As in variation in higher organisms, the observed changes as a rule leave a majority of characters unaltered.

The use of language concerned in the classification of fungi and higher plants such as *genus* and *species* implies an ability to distinguish and define large groups and small groups, and to state with some degree of assurance the probable evolutionary or genetic relationship of the units concerned. The number of constant morphological points of difference available for purposes of classification are far fewer among bacteria than in the higher forms, and for purposes of taxonomy, physiological characters have been largely used. Though these have to some extent been used for the same purpose in general botany and zoology, morphology has almost universally taken precedence in those sciences and functional peculiarities or characters, without demonstrable structural changes, have been regarded as more subject to temporary and fluctuating changes. Nevertheless, certain well-marked differences in form and staining properties separate in a natural way large groups of bacteria. Such characters, which justly have been given value for the formation of families or orders, are the morphological features which separate staphylococci, streptococci and meningococci, and these from the two large groups of Gram-positive and Gram-negative bacilli. Again, the ability to form true endospores or flagella are characters of importance which show a relationship between some of the groups in which these properties occur. Among functional characters which are very stable and available for this purpose in some cases, are the oxygen requirements (obligatory aerobic or anaerobic growth). The fermentation of certain sugars is also, in many instances, a character of very great stability, e.g. the power or inability of many species in the Gram-negative intestinal group to ferment lactose, the fermentation of glucose and its refusal of cane-sugar by the diphtheria

bacillus and other similar characters. Even in such fairly constant characters variations in special instances can be demonstrated.

When an attempt is made to delimit smaller groups corresponding to genera and species, the essential morphological characters can seldom be defined and reliance has to be placed on physiological data. The criteria relied on are of less generally recognized applicability than in higher organisms, and the distinctness depends on observed constancy and persistent pure breeding, more than on the existence of well-marked and obvious peculiarities of acknowledged and universal ' species value '.

Thus, motility has been often considered a valuable aid in separating large classes, but closely allied species which might otherwise be considered almost identical, may be non-motile and motile respectively, e.g. *B. mallei* and *B. whitmori*, or *B. pestis* and *B. pseudotuberculosis rodentium*. On the other hand, motile and non-motile races may occur as the offspring of the same bacterium as in the H and O forms of *B. proteus*, *B. typhosus*, &c.

Such a character as the ability to produce acid or gas from a sugar in some cases appears to be a valid reason for separating two species, e.g. lactose or mannitol in the *B. typhosus* and *B. dysenteriæ* group, but in other instances this character varies in undoubted members of the same species or even in variants of the same strain, e.g. the variants of *B. coli* (Neisser ; Penfold and Revis) and *B. typhosus* (Twort, 1907) and *B. acidi lactici* (Arkwright, 1913). Again, reactions such as agglutination with specific antiserum may not be constant within the species (e.g. H and O agglutination of *B. proteus*, though of great value in other cases for uniting species which exhibit striking differences of other kinds, e.g. forms of *B. dysenteriæ* which produce acid from cane-sugar, or non-motile or lactose-fermenting variants of *B. typhosus*, &c.

In fact, as is the case among allied species of higher organisms, the definition of a species has to include a description of several characters, not of one alone ; and the conventional view of a bacterial species which has been more prevalent of late years—that assured specific difference may depend on differences of function and on the observed constancy of one or two such criteria, as in streptococci and salmonella—cannot be regarded as entirely satisfactory.

The not infrequent uncertainty in definition of species in the higher multicellular plants and animals, and the separation of species from varieties or micro-species, on the one hand, and from genera, on the other, has led some botanists to claim that a species cannot be defined, except genetically, i.e. by a method which involves observation of several generations. This process corresponds in bacteria to the successive survey of enormous numbers of generations of bacteria in a relatively small number of subcultures, usually in mass culture. Other botanists deny that the idea of a species is capable of absolute definition, but claim that it is rather an expression of convenience for purposes of nomenclature and a rough statement of general relationship.

A similar discussion among bacteriologists has led to no greater consensus of opinion. The writer holds the view that the insensible merging of one so-called species of bacterium into another sometimes occurs, whilst, for purposes of convenience, definite species, though at present imperfectly defined, may be accepted provisionally.

The use of the term ' pure line ' in bacteriology for the descendants of a single bacterial cell does not appear to be very happy, since, although undoubtedly different forms or races may be separated from apparently pure cultures, these are not exactly comparable to Johanssen's ' pure lines ' in beans and wheat. In the first place, the different forms often arise again readily from the ' pure lines ', and any resemblance as regards natural and artificial crossing cannot be verified, since conjugation in bacteria cannot be assumed to occur. At any rate, the occurrence of syngamy and conjugation cannot be observed at will, have never been satisfactorily demonstrated, and the evidence in their support is very slight.

One of the chief difficulties in classifying bacteria is due to the constantly increasing records of variation and the imperfect knowledge at present of the range of this variability for a single culture. Before entering more fully into the subject of variation it will be necessary to state the kinds of variability which will be considered, and the different changes which have been included under this term.

The Meaning of the Term Variation. The Kinds of Change included in the General Term Variation.

The changes included in the general term variation may be divided into (1) Temporary changes or modifications, and (2) Hereditary variations, under which headings will be included many reversible variants, the so-called ' cyclostages ' and the results of ' dissociation ' as well as the so-called ' mutants '. These headings are not mutually exclusive and are found to overlap. It is with hereditary variations that this chapter is chiefly concerned.

Temporary Variation, Modification or Fluctuation.

By this is meant any alteration in morphology or mode of growth of individual bacilli or of their colonies, or in physiological and biochemical behaviour, which, as far as can be ascertained, is *not hereditary*, that is, is not passed on by subculture unless the special conditions associated with its appearance are maintained. Such characters may be, and usually are, obviously associated with environmental conditions, such as the composition of the culture media, the temperature, &c., and are recognised by their immediate disappearance on the return to the typical or previous state, when the original conditions are restored. The observed number of ' modifications ' or ' fluctuations ' is very great. Amongst the most marked which may be mentioned are morphological changes and biochemical fluctuation.

Morphological Changes.

Length, shape, size, &c. In this group may be placed: increased length of the individual bacilli or appearance of giant forms, e.g. of *B. coli* grown in strong salt solutions; growth in very long thread forms; unusually long and clubbed forms of certain Corynebacteria, especially in old cultures; growth in very long chains of streptococci in liquid media, and of *B. anthracis* in blood; growth in long rod-like forms or as very short coccal forms of many strains of *B. coli*, *Br. melitensis*, &c.; irregularity and great variation in size, e.g. in rather old cultures of meningococci and gonococci; great differences in size and shape, including the large irregularly stained forms found in cultures many days old or in the tissues of animals dead of an acute infection, e.g. *B. pestis* in smears of the spleens of rats. The large and irregularly shaped micro-organisms found under such circumstances have been called 'involution' forms, but they are often viable and there is not much evidence that they are degenerating on the one hand, or unusually resistant (arthro-spores) on the other. The giant, irregular, forked and segmented forms found in bodies of dead animals are temporary variants due to a special, and, no doubt, often hostile environment. The rare occurrence of branching and the three-point growth of Gardner (1925) may be included under this heading.

Almquist (1904) and Kuhn (1921) described similar unwonted forms of *V. choleræ*, *B. typhosus*, &c., grown in garden soil, especially very slender filaments and large globules, and attributed to them special reproductive functions which have not been accepted by the majority of subsequent workers. The extraordinary abnormal or unusual forms of *B. dysenteriæ*, &c., found in cultures made in strong solutions of special salts by Hatas, Eisler, &c., belong to this category of temporary environmental modification. The large rounded 'citron' forms seen in many anaerobic cultures of sporing anaerobes and many of the aberrant forms described by Lõhnis and Smith and by Mellon and others, though regarded by them as important phases in a life-cycle, are more probably also fluctuants or modifications produced by a special and temporary environment, and not essential sexual or specially reproductive stages in a life-history. This is rendered more probable by the fact that the same bacteria can be cultivated for long in many different media and in the animal body without these forms arising, and that the so-called reproductive or special forms are irregular in the time and order of their appearance, often of very bizarre appearance, and at other times too varied to escape the suspicion of contamination with alien growths. If special reproductive forms really occur in pathogenic bacteria, an acceptable method of demonstrating their occurrence has still to be devised.

Haag (1927) has also described a complete cycle (cyclode, Enderlein) in *B. anthracis*, including two forms of gonidia, one Gram-positive and one Gram-negative, symplasms, motile, Gram-negative rods, sporing and non-sporing and filter-passing forms. His work is open to the criticism

that the changes which he described chiefly occurred in extracts of decomposing material which had only been sterilized by filtration. Since the 'cyclode' included a filterable form, his precautions to avoid contamination would not seem to be sufficient on his own hypothesis.

The method of reproduction described by Thornton and Gangulee (1926) by which one large cell divides into numerous small forms sometimes called gonidia, has not been accepted as yet for pathogenic bacteria, though such methods of multiplication are claimed by Löhnis and Smith for a very large number of pathogenic and non-pathogenic species.

If the special forms or so-called 'cyclostages' become '*fixed*', they enter another category as hereditary variants and will be considered again later.

Staining. Here may be grouped: changes in composition and structure as revealed by staining, such as the presence of Ernst granules in Corynebacteria on special culture media; variations in resistance to decolorization by alcohol after Gram-stain, or by acid after Ziehl-Neelsen, which depend on the culture medium and the age of the culture or on conditions inside the animal body. In some cases the enlargement or formation of a capsule by streptococci and pneumococci or *B. anthracis* in the animal body, or in presence of serum or other media, may be also quite temporary, but in other cases the presence or absence of a capsule appears to signify a more permanent and hereditary change.

Colonies. Variations in the size and shape of colonies are very often mere modifications dependent on the content of the culture medium, and especially on the moisture and alkalinity, &c.

Flagella. The growth of flagella at a certain temperature and not at another, or at any rate, their partial suppression, is very characteristic of certain bacteria, e.g. *B. pseudotuberculosis*, *B. inconstans* and some strains of *B. proteus*, *B. typhosus*, &c. Defective growth of flagella and loss of motility have been shown by Braun and Schaeffer (1919) to accompany culture on carbolic agar (0·1 per cent.), and agar deficient in nutritive material was found to have the same effect.

Though many of these changes affect important structures and properties of the bacteria, they are, nevertheless, often quite temporary and only last as long as the special environment.

Biochemical Variation.

Other modifications affect more obviously the biochemical activities such as (i) the production of pigment, which is often less in cultures grown at 37° C. than at 20° C. (e.g. *Staphylococcus aureus* and *B. prodigiosus*); (ii) production of indole, which is influenced by the pH of the culture media, since it is not formed, or in much less amount, by *B. coli* in the presence of glucose or lactose or undue acidity from other cause; (iii) toxin is not formed by *C. diphtheriæ* or is rapidly destroyed at pH 6·6, but is formed readily at pH 7·6 in broth which is otherwise the same.

Similar reactions prevent the formation of hæmolysin by *Staphylococcus* and *V. choleræ ;* other peculiarities in composition of the medium also have an important influence.

Since all the instances of changed appearance or behaviour of cultures mentioned above are due to the particular environment in which the cultures are placed, and since they cease when a subculture is made on to a fresh and suitable medium, such changes or variations are classed as fluctuations or modifications. It is noticeable that some of these temporary forms are exactly the same as true hereditary variations in direction and even in extent. This fact may be used as a cogent argument in support of the hypothesis that hereditary variation is produced in bacteria by environmental conditions.

A clear idea can seldom be formed of the truly natural or standard environment of a pathogenic bacterium, and the so-called ' normal ' culture, i.e. that recently grown from the animal body on ordinary laboratory culture medium, might rather be regarded as a ' modification ' due to the conditions to which it is being subjected, in so far as it may readily change if the medium is altered. Nevertheless, such a culture has a fair claim to be called normal, if its characters remain constant on an ordinary culture medium, the growth is rapid, the individual bacteria are fairly uniform and the antigenic components appear to be complete.

Hereditary Variation.

Introduction.

The criterion of hereditary variation is the persistence of the new character in culture media and under conditions in which parallel cultures of the variant and of the original form yield distinctive and characteristic growths. Similar constancy may also sometimes be demonstrated after passage of the variant through the animal body. The critical test is not quite so easily applied as at first sight appears, since many hereditary variants tend to revert to the original form after a few subcultures (which may mean thousands or millions of generations of bacteria) or even in a few days in the same culture, unless special precautions have been taken to isolate the variant and to obtain it free from admixture of parental factors in its composition. Nevertheless, certain variants can be obtained pure in this sense and may then retain their peculiar features for very many subcultures, even on varied media.

Several writers have attempted to distinguish two kinds of hereditary variation. The one called mutation by Neisser (1906), Massini (1907) and Müller (1911), on account of its supposed resemblance to the ' mutations ' of De Vries, was characterized as making a sudden large and irreversible change. The other displayed a slight, but sometimes progressive change. Baerthlein claimed that no distinction between small and large hereditary variations could be made ; this seems to be the general view and coincides with the tendency among geneticists regarding higher

organisms. Reversion is observed to occur in many variants, but when the variant bearing the new character has been repeatedly purified by plating out and selecting single colonies, a fixed form can often be obtained which is pure in composition and does not relapse when grown on a standard medium under standard conditions. These two stages probably correspond to Toenniessen's (1921) ' alternants ' and ' mutants ' of Friedländer's bacillus. It is a question whether any variant is completely irreversible. It has been urged against the use of the term ' mutant ' in bacteriology that in the phraseology of genetics of the higher forms of life the term is held to imply a definite change in a chromosome. Such a definition would here be unsuitable, since, in bacteria no chromosomes have been observed and genes are purely hypothetical. It is, therefore, probably best to avoid the term ' mutant ' in bacteriology. The hereditary variations here described correspond to the ' Dauermodificationen ' of Jollos (1924), and include the so-called ' mutants ' and ' alternants '.

The Characters affected by Hereditary Variation.

Morphology, Capsule, Spores.

Hereditary variation may affect independently many characters in bacteria so that several different variants may be obtained in artificial culture from a single strain. Thus, Baerthlein (1918) obtained from a single strain two different races of *B. pyocyaneus* which grew (1) in opaque, dry, crumbling, colonies of short, wide, bacteria, and (2) in moist, translucent, smooth-edged, colonies of long, slender forms. From each of these he obtained three further variants, (*a*) in colourless colonies, (*b*) light green, forming fluorescin but no pyocyanin, and (*c*) dark green, containing fluorescin and pyocyanin. So that six variants resulted from the selection of colonies. Some strains are more liable to vary in certain directions than others of the same species. The natural tendency of species to vary is exemplified by the fact that different strains of presumably the same species which have been isolated from different sources, e.g. *B. typhosus* from different cases of typhoid fever, have often a certain individuality by which they can be distinguished (Felix and Olitzki, 1926). These features may merely concern a tendency to vary or may affect special properties. Some of the characters chiefly affected by hereditary variation will now be shortly reviewed.

Morphology : Size and shape, length, formation of threads or chains, roundness of the ends, clubbing, possession of deeply staining granules, segmentation and irregular staining, as in diphtheroids; degrees of ability to retain Gram and Ziehl-Neelsen stains, the latter especially in the non-pathogenic mycobacteria. The presence or absence of flagella is a common feature of variants in the *B. coli* and *B. proteus* groups and the vibrios.

Capsule. The presence or absence of a capsule is a well-known form of variation in the pneumococcus and the bacillus of Friedländer (*B. pneumoniæ*) in which species the capsule is associated with important antigenic and infective properties. In *B. anthracis* capsulation has been

studied with much detail by Preisz (1911), Fischoeder (1909) and others. Although believed formerly to be an important condition of virulence, it is now regarded by some authors as a fugitive character due to direct action of serum on the bacillus and to be of no great pathogenic importance in the latter species.

Formation of endospores. In species of bacteria which form endospores, hereditary variants which do not form spores have been repeatedly observed. Roux (1890) described such a variant of *B. anthracis*. It was obtained most readily by growing the sporing form in broth containing 1 in 5,000 to 1 in 1,600 phenol. The asporogenous form resembled the sporogenous very much in the appearance of the cultures. Its virulence could be raised by passage through susceptible animals, but nevertheless, it remained asporogenous while under observation. Rosenthal (1926) obtained a similar asporogenous form of the same organism by growth of the sporing form in filtrates of *B. anthracis* cultures.

A. H. Gee (1927) has recently recorded an asporogenous form of a bacillus, which he calls *mesentericus-vulgatus*, found in haddock. The forms which he described cannot, however, be definitely accepted as derived from a pure culture without confirmation.

Filterable forms. Individual bacteria of much smaller size than those usually seen in cultures no doubt occur and these are sometimes sufficiently small to pass through a Berkefeld or other type of filter which retains the vast majority of bacteria present. The permeability of a filter has been shown to depend on the conditions under which it is used. Filtration of a large quantity for a long time, under high pressure or very rapidly, are conditions which have been found to result in bacteria or other particles passing through which are retained under other circumstances. Further, the number of bacteria in the suspension influences the result. A filter may be sufficiently effective to retain a very high proportion of the bacteria present but not necessarily all. For instance, 1 in 10,000 or 1 in 100,000 may find a way through, and the larger the number exposed to the chance of escape, the greater the likelihood that an occasional viable individual will pass through.

It has, however, been maintained by a certain number of workers that extremely minute forms of bacteria often occur in cultures, especially old cultures, and in effusions or excretions of the animal body and that these readily pass through Berkefeld or even porcelain filters. Löhnis (1922), Löhnis and Smith (1923), Mellon (1919–25) and others regard these forms as phases in the life-cycles which they describe. Hort (1916, 1920) believed that such very small living forms arose from *B. typhosus* and other bacteria. Friedberger and Meissner (1923) and Friedberger (1926) maintained the existence of the ' kryptogenic vira ' which were living, invisible, uncultivable forms, derived from the organs of infected animals, and they described the passage in series of such descendants of *B. typhosus* through guinea-pigs. Ultramicroscopic forms of pathogenic bacteria are not generally accepted by bacteriologists as a definite phase or

stage in their life-history. However, a considerable number of workers (Fontes, 1910, 1923; Vaudremer, 1923; Hauduroy and Vaudremer, 1923; Valtis, 1924) claim to have demonstrated a filterable living form of *B. tuberculosis*, which can multiply *in vitro* and in the animal body. Calmette (1928) has recently summarized and favourably reviewed the evidence. The nature of these minute forms and the part which it is claimed that they play in pathology are not at present at all clearly defined. They are said to occur as granules which multiply in the animal tissues and produce enlargement of lymph glands in which a few ordinary tubercle bacilli can sometimes be recognized. Tuberculous lesions do not occur, and even after passage to other animals typical tuberculous changes are very rare.

Living filterable forms of *B. dysenteriæ* (Shiga) were described by d'Herelle (1922) in filtrates of cultures lysed by bacteriophage. Twort (1920, 1922) recorded, but did not claim viability for the granules which he saw in lysed dysentery cultures. Hauduroy (1924) described similar products as the result of the action of a lytic agent on *B. coli*, *B. typhosus*, *B. dysenteriæ* and *M. aureus*. Fejgins (1924) also claimed that a filterable stage of *B. proteus* OX 19 could be obtained, and related it to the virus of typhus fever. It has also been suggested by Nicolle (1925) that bacteriophage is perhaps a viable inframicroscopic form of bacteria. Hadley (1927) also considers the filterable forms and phage to be closely related in the process which he calls dissociation.

Growth in Broth.

Variations in the manner of growth in broth and in other liquid media are striking phenomena. The character, in virtue of which certain cultures, especially of the *B. coli-typhosus-dysenteriæ* group, make granular growth in broth and are clumped in salt solution (0·85 per cent. NaCl), has been observed for many years (Savage, 1901 ; Nicolle, 1898) under the name of spontaneous agglutination. This property formerly acted as a bar to testing the agglutination reactions of these cultures. The deposit in these cultures is widely scattered over the concave bottom of the test-tube instead of being collected in a small round spot, as occurs when the normal diffuse, turbid growth gradually forms a sediment. That the granular growth is due to the agglutination by salt is shown by subculture into broth containing a lower concentration of salt, when diffuse growth occurs, rendering the broth uniformly turbid as in the normal cultures.

The spontaneously agglutinating variants of the *B. coli-typhosus-dysenteriæ* group are usually distinguished by the appearance of their colonies on agar and by a marked change in their serological properties. Such cultures correspond to the rough or R type of variant (Arkwright, 1920, 1921). They may be either motile or non-motile if they belong to a species which is usually motile.

In spontaneously agglutinable broth cultures there is very commonly a film on the surface which readily breaks up and falls to the bottom of the tube, thereby increasing the deposit. If the culture is inadvertently

shaken, the surface film may not be detected, but only a ring of culture, adherent to the glass at the upper limit of the liquid. A surface film is usually, but not always, present in R cultures. On the other hand, a film may be present in quite smooth cultures and its presence may in part be due to a specially aerobic tendency of the culture.

The formation of granular rather than turbid growth and of surface films in broth is characteristic of some species of bacteria, e.g. *C. diphtheriæ*, and other species of the Corynebacteria. Variants of such cultures occur of which the broth cultures are more or less granular than normal, and sometimes quite diffusely turbid variants may be obtained. Not infrequently, the granular cultures, though due to spontaneous agglutination, do not appear to be especially influenced by the salt content of the medium, but retain this character although the percentage of salt is very much reduced. In these cases there is often no evidence that the serological properties are altered, as they are in the R variants above mentioned.

The variant of *B. lepisepticus* described by De Kruif (1921) as the G form on account of the granularity of the broth cultures, in contrast to the diffuse turbidity of the broth containing the normal or D form, was spontaneously agglutinable in salt and also showed antigenic change. In these respects and also in its low virulence the G variant resembled the R form of *B. typhosus*. Other kinds of variants which also form coherent surface films commonly occur in the Pasteurella group.

In some broth cultures clearing of the liquid with a large deposit is brought about in another way. Quite apart from any special tendency to spontaneous agglutination due to salt or other causes, a coherent, wrinkled, film grows at the surface of the broth and after a day or more breaks up and falls to the bottom of the tube. This kind of culture may occur as a variant of a normal turbid culture of Salmonella or *V. choleræ*, but is more often seen in strains of Pasteurella and some other families of bacteria. Such a surface film may be so coherent that it is only emulsified with great difficulty ; a wrinkled film is seen in cultures of one form of *B. subtilis* (Soule, 1928) ; and is described by Gee (1927) in a form or variant of *B. mesentericus-vulgatus*. This form of growth appears to be associated with the production of some glutinous material on the surface of the bacteria. It is not necessarily associated with the antigenic change found in the R form of *B. typhosus*, &c., but the bacteria are usually non-motile, and may be deprived of the flagellar antigen (see the account of variants on agar, p. 324).

Another variant which may be associated with a clear broth culture, and a large, rather widespread deposit, that at first sight suggests the spontaneous agglutination of an R variant, is seen occasionally in cultures of *B. typhosus*, *B. paratyphosus* or *B. coli*. Its special properties are due to the growth of the bacteria in very long threads which are quickly deposited without agglutination. The serological properties may be characteristic of the S or normal form (cp. the form of *B. subtilis*

described as R by Soule, 1928). A similar variant is common in the streptococcus group, and is due to the growth of long chains, especially if these form compact tangles. In both cases the coils of long chains or threads can be detected in the structure of the colony with a suitable magnification. These long forms are sometimes, but not necessarily, associated with recognized alteration in other characters.

Change in the character of the broth culture so that it is of a viscid or slimy consistency is another common variation in *B. coli*, *B. dysenteriæ*, &c., and is often hereditary. It may be associated with a sticky growth on agar, but it is not very noticeable in cultures from well-marked mucoid colonies on agar.

Variation in production of pigment is often well seen in broth cultures of *B. pyocyaneus*, *B. prodigiosus*, &c. ; the pigment may be different in degree or entirely absent in variants, or it may be considerably altered in shade without being lessened in intensity. It has often been noticed by the present writer that old cultures of R variants of the coli-typhoid group which have been made for some weeks have become a deep brown colour, while the S cultures of the same strain have retained the original shade of the uninoculated broth. The nature of the chemical change responsible for this increased pigmentation has not been ascertained.

Growth on Solid Media.

The characteristics of the growth on solid media, especially agar and gelatin, are of particular importance in the study of variation, because cultures of some species can be readily recognized by their appearance on solid media, and the descriptions of different species of bacteria have been largely concerned, more especially in the earlier days of the science, with the shape, colour, translucency, &c., of the colonies. For purposes of classification of late years less reliance has been placed on such characters, since it has been recognized how much they depend on slight differences in the medium, such as dryness, and on the juxtaposition of similar or diverse colonies, and since the occurrence, even in pure strains, of well-marked variants indicated by the changed appearance of the colonies, has been repeatedly observed. The study of variation by selecting colonies of differing aspect was very much developed by Baerthlein (1912). By his technique of picking off well-isolated colonies of different appearance from agar plates, it has been found possible to obtain many hereditary variants. When a sugar and an indicator for acidity are added to the medium, as in the neutral-red-bile-salt-lactose-agar of MacConkey, Endo's sodium-sulphite-fuchsine-agar, or the methyl-violet-agar of Conradi and Drigalsky, it is possible to detect and pick off as single colonies, and in this way to isolate pure variants which are altered in respect of the fermentation of some particular sugar.

Most of the variants which have been studied in pure culture show their special peculiarities in their colonies on plates of nutrient agar, either alone or containing some additional substance such as blood,

carbohydrates, alcohols, &c. The following hereditary changes can be detected by the growth of colonies on simple nutrient agar and so can be isolated, but many other variants are seldom or never obtained pure, because they cannot be detected in this way.

Variants detected by the Form of the Colony.

The characteristic appearances of variant colonies can in certain cases be definitely correlated with some other character such as motility, capsulation, agglutinability, &c.

H and O forms.

The growth on an agar plate may show whether the culture is motile or non-motile. Confluent growth, especially when in the form of a very thin film or '*hauch*' on the surface of the agar, as in cultures of ordinary *B. proteus*, may indicate that the bacteria are motile, and isolated colonies of these strains under the same conditions usually consist of non-motile bacteria. This distinction was described by Firtsch (1888). Weil and Felix in 1917 attached great importance to the difference between the two forms which they detected and isolated by this manœuvre, and which they called the H (*hauch*) and the O (*ohne hauch*) forms. The O or non-motile form of *B. proteus* usually forms round, smooth, domed colonies, distinguished by their isolation and the absence of a thin spreading margin. Non-motile, smooth colonies of *B. typhosus*, Salmonella, &c., can sometimes be distinguished from smooth, motile colonies by slight differences in shape or translucency. These two forms have proved to be hereditary in some instances. Similar variants, which can sometimes be detected by similar means, have been found in many other species, especially in the coli-typhoid group and also among the sporing anaerobes such as *B. tetani* (Fildes, 1927; Felix and Robertson, 1928). Highly motile strains or variants can sometimes be obtained pure from mixed cultures by subculturing from the spreading edge of growth which ascends the moist surface of an agar slope (Gorini, 1903, 1917, 1928; Choukévitch, 1911; Fildes, 1927).

Mucoid variants.

Mucoid variants of *B. paratyphosus B.* and *B. dysenteriæ* described by Müller (1910), Fletcher (1918), Trawinski (1923), and P. B. White (1926) can be readily detected on agar by the outer zone or sometimes the whole colony becoming swollen, translucent and glairy. The mucoid material may become liquid so as to flow to the lower part of an agar slope. In order to demonstrate this kind of change, the agar must be moist. The change appears especially when the culture, after growth for 24 hours in the incubator, is kept at room temperature or in the cold-room. The mucoid degeneration or change does not appear to have any intimate relation to change in the antigenic structure, but motility is absent and agglutination with serum is usually very feeble; in many cases the mucoid

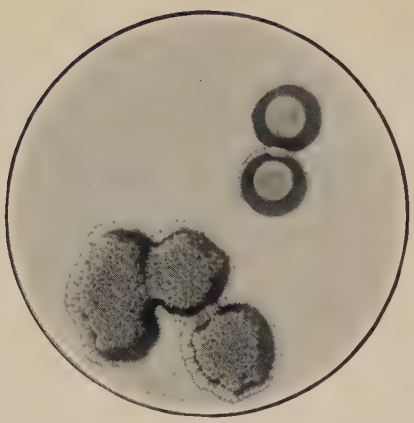

FIG. 1. Smooth (S) and rough (R) colonies of *B. typhosus*.

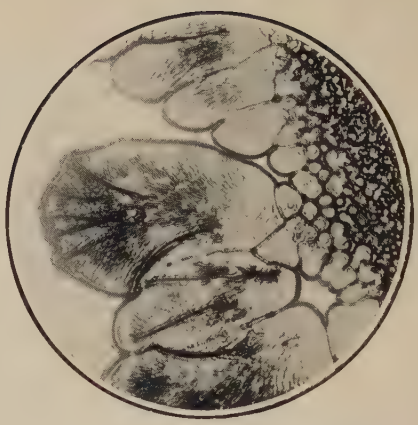

FIG. 2. Part of a growth of confluent, wrinkled colonies of Salmonella, with 'S' antigen.

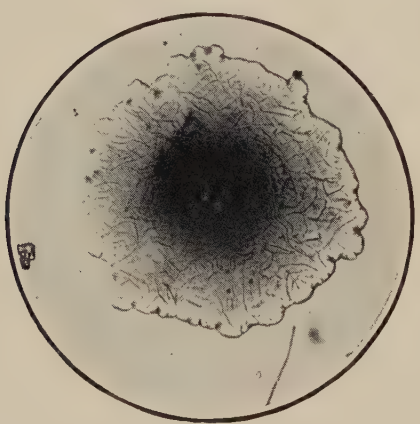

FIG. 3. Veined colony of *B. typhosus*, containing many threads and chains, with 'S' antigen. × 22.

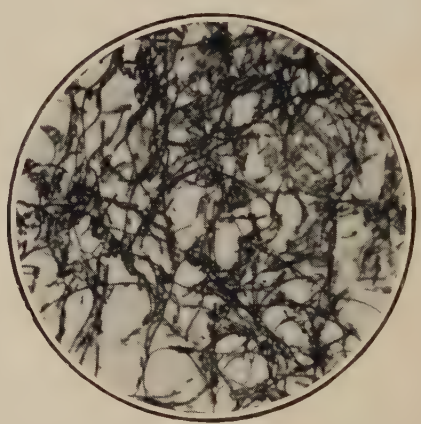

FIG. 4. Stained film from colony in Fig. 3, showing tangle of threads. × 250.

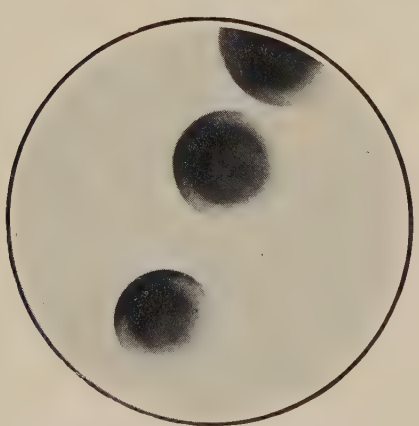

FIG. 5. Thick, domed, scarcely granular colonies of *C. diphtheriæ*.

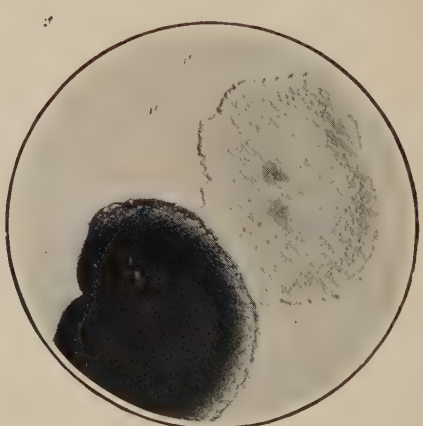

FIG. 6. (*a*) Thick coarsely granular and (*b*) thin coarsely granular colony of *C. diphtheriæ*.

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change appears to be only a temporary modification, and the culture returns to normal on subculture, or partial reversion occurs as shown by the appearance of some normal colonies.

Smooth (*S*) *colonies* (see Fig. 1).

These are typically round, domed colonies, with a shiny surface, which are usually translucent and have only very small granules as seen with a low power of the microscope. The margins are even and regular in young cultures; but minor differences in shape and appearance occur in the various kinds of bacteria. The growth in broth, especially in the *B. coli-typhosus* group, is uniformly turbid. The component bacteria may be motile or non-motile, but the somatic antigen is well developed. This is the normal form usually seen in recently isolated and regularly subcultured strains of the Gram-negative intestinal group. Colonies of similar appearance occur in other families of bacteria but are not always analogous in other respects.

Rough (*R*) *variants* (see Fig. 1).

Irregularity of the surface and margin of a colony may give an indication of several different kinds of variants, but the commonest and most important is that described as 'rough' by Arkwright (1920, 1921) in the *B. coli-typhosus-dysenteriæ* group of bacteria. Typically the colonies are thin, flattened, irregular in surface and outline, and are coarsely granular in structure, as seen with a hand lens. They are often larger than the S colonies, but may be very small, and in either case are usually especially thin at the edges and slightly raised in the centre. The component bacteria have several characteristics which distinguish them from the S form. Another form of colony of the R variant is flattened but not so thin, is slightly raised with rather irregular margin and has a dull but less irregular surface. It is more opaque than the S colonies, but of about the same thickness, and is seen to be coarsely granular under a low power of the microscope.

Broth cultures of the R form are granular and a deposit soon forms, leaving a clear supernatant. The bacteria may be motile or non-motile. There is often a surface film which is very fragile. Emulsions from agar, like the broth cultures, are spontaneously agglutinable in salt solution (NaCl 0·85 per cent.), but can be emulsified in distilled water or weak solution of sodium chloride (0·4 to 0·1 per cent.).

The irregularity of the surface of the colony and its granular structure are apparently due to the bacteria forming clumps as they multiply, and increasing from many centres instead of spreading out uniformly as in the S colony.

There are many intermediate grades between the typical R colonies and the S, due to the characters of the component bacteria not being alike, or to the change from S to R, which is occurring in the culture, being gradual, so that the individual bacteria are not fully changed at first.

The serological character of the R variant is altered as regards its somatic antigen, so that if the flagellar antigen is absent it no longer reacts with the S antiserum, but has acquired peculiar properties of its own. P. Bruce White (1927, 1928) has shown that the spontaneous agglutinability can be removed by extraction with alcohol, and has also pointed out a tendency of the unextracted bacteria to agglutinate with normal serum, if this is not much diluted.

The R forms of *B. typhosus* and *B. paratyphosus* are very much less virulent than the S. Cultures possessing the distinctive serological and pathological properties of the R form in the intestinal group sometimes grow in colonies very like the S form, and are then little, if at all, agglutinated by salt. This anomalous behaviour is explained by White as being due to a low content of the lipoids which are essential for agglutination. The composition of this form of R is then like a typical R after extraction with alcohol. Its peculiar antigenic properties are the same as those of the typical R form, but it makes a stable emulsion in salt solution (Goyle, 1926).

A variant of the pneumococcus was first described by F. Griffith (1923) as the R form of this organism, and was shown by him to be non-virulent. It is similar in some respects to the R forms described above. It is characterized by dullness of the surface and altered agglutination reaction with specific serum. It is apparently identical with the R or ' degraded ' variant of pneumococcus described by Reimann (1925, 1926, 1927), who showed that it had lost its capsule and was not only devoid of virulence, but was no longer distinguishable by agglutination from R pneumococci of other types. The R pneumococcus is not markedly agglutinated in salt solution.

Colonies composed of long rods, threads or chains (see Figs. 3 and 4).

Medusoid colonies.

Colonies of *B. coli*, *B. typhosus*, &c., which show lines like the veins of a leaf when examined with a hand lens or microscopically can, as a rule, be seen to contain unusually long bacillary forms, which may persist through a number of subcultures. This variant probably shows a tendency towards the more complete and persistent variant next described.

In many different groups of bacteria variants may appear in which the substance of the colony is seen, under a low power of the microscope, to consist of long threads or chains running partly in parallel strands but coiled and twisted and often projecting in loops beyond the margin. The surface of such colonies is often very irregular and knotted, but may nevertheless be quite shiny. A well-known example of this is the ' medusa-head ' colony of anthrax, but very similar colonies occur as variants in *B. subtilis* and occasionally in *B. typhosus*, *B. coli*, &c. They are also often seen in cultures of streptococci, where the growth is in long chains, but in this latter group the coils and loops are often so tangled and irregular that their detection in the small colony requires a higher magnification.

The serological properties of medusoid colonies of *B. typhosus* and *B. paratyphosus* may be the same as of the normal S colony. In the case of *B. subtilis* the antigenic structure may be altered (Soule, 1928), and N. C. Graham (personal communication) finds a flagellar antigen which varies in amount in different variants. Streptococcus colonies of this type may show differences in serological behaviour and alterations of virulence, but the medusoid character is primarily due to the presence of long chains and does not necessarily indicate any other special change.

Coherent colonies (see Fig. 2).

Variant colonies of a coherent texture occur in a number of different groups of bacteria. They have irregular surfaces and uneven margins. The surface is often wrinkled and the consistency of the colonies dry and very coherent, or with a tendency to break up into large fragments. This type of colony corresponds to the broth cultures with coherent and wrinkled surface films. It seems to indicate a firm, sticky, outer layer on the bacteria. Motility is, as a rule, absent, and sometimes, at any rate, the corresponding flagellar antigen also is deficient. For instance, the wrinkled colonies of the ' O ' form of *V. choleræ* studied by Balteanu (1926) contained the same smooth somatic antigen as the normal cultures, and an emulsion was not agglutinated by salt. A culture of *B. paratyphosus B* given to the writer by Mrs. Barratt grew in firm wrinkled colonies on agar, but contained normal S antigen, and was in no sense ' R ', except in the irregularity of the surface of the colony. Variant colonies of a similar kind have been observed in *B. ærtrycke* and *B. pseudotuberculosis rodentium*. Perhaps the so-called ' R ' colonies of *B. tuberculosis* are of the same kind.

These and similar variants have been placed by some writers in the same category as the rough form of *B. typhosus*, &c. (Gee, 1927), but the evidence that there is much resemblance except in the uneven surface and outline of the colonies is not convincing.

Irregular and ill-defined colonies.

Many other kinds of variant colonies have been described, especially by Baerthlein (1912, 1918) and Eisenberg (1914), in different species. Some of these, when picked off a plate culture, yield a pure race of the variant type of colony, but have no other characteristics by which the variant can be recognized. Very many other unusual-looking colonies when replated yield growth which cannot be distinguished from the normal. The latter occurrence is explained either by a rapid reversion or by the first abnormality of the colony being merely due to chance conditions on the plate.

The selection and subculture of colonies of special appearance and containing variants, leads in many instances to a growth on an agar slope which is very similar to the first variant colony. Usually, however, when an emulsion from the slope is spread on a plate the colonies are seen to be again mixed in character. It is then only by repeated plating and selection that a variant yielding uniform colonies can be obtained. When

this stage is attained it is often well, in order to be sure of purity of constitution, to plate an unclumped broth culture or emulsion and select again at least two or three times. When at last uniformity of colony has been obtained the culture is much less liable to reversion and many remain constant for a long time. In most of such instances the effect of plating repeatedly and selecting is to eliminate those bacilli which form colonies of mixed character and to obtain a culture from one of uniform constitution. The individual bacilli in the earlier stages no doubt vary in their content of the new factors. In the case of many types of variants complete purity is never attained, reversion seems to be constantly occurring or the variant may disappear, being represented in fewer colonies or none after two or three attempts at selection and subculture. As an instance of the varied behaviour, stability or instability of different variants, may be cited the experiments of Baerthlein (1912) and Eisenberg (1912) with *V. choleræ*. They obtained numerous types of colony but most of them were very unstable, though two or three remained very fairly constant. Besides the differences in appearance in the colonies and some differences in morphology and in growth in broth, in most instances they did not obtain any notable change. Variants whose differentiation affects only a single character are especially liable to reversion.

Balteanu (1926) described a variant of *V. choleræ* which was very probably identical with one of Baerthlein's. This variant, which he selected chiefly by its most obvious character of opacity, was very firm and coherent in consistency. It remained fairly constant as long as it was grown on agar, but had a tendency to revert in broth. Balteanu showed that this variant had agglutination properties which were analogous to the O form of *B. proteus* of Weil and Felix. It appeared to have almost entirely lost flagella, and consequently in agglutination reaction only displayed the properties of the heat-stable, small-flaking antigen, having lost the heat-labile, flagellar antigen, which Balteanu had demonstrated in the normal form.

Dwarf colonies.

Dwarf colonies of *V. choleræ*, *B. dysenteriæ*, &c., have been described by Baerthlein, Eisenberg and others. They are generally of dense consistency and subcultures from them grow feebly. They appear often merely to express a low degree of vigour.

Lytic colonies.

Twort (1920, 1922) described special forms of *B. dysenteriæ* (Shiga) arising most frequently in cultures grown on maltose agar. They were distinguished to some extent by the appearance of the colonies, and had a tendency to spontaneous agglutination, but were particularly remarkable on account of the length, large size, irregularity in shape and staining properties of the individual bacteria. The rods contained many granules which were also present free in the culture medium. Twort stated that these special forms were dissolved more readily than the normal by a

lytic agent which was also present, but that when isolated and grown further they became more resistant to lysis. The exact relation of the special forms to the lytic agent was not established, but Twort's lytic agent is generally recognized as identical with the bacteriophage of d'Herelle.

The 'notched', 'nibbled' or 'moth-eaten' colonies resulting from action of bacteriophage are well known. Though much work has been done with colonies completely or partially resistant to bacteriophage, the work is very difficult and the results are apt to be inconsistent, owing to the persistence of the phage in culture and its latency, reappearance or persistent action. Some of the more definite results of phage action in producing changes in a race of bacteria are described in Volume VII.

So-called 'suicide-colonies' have been described by Hadley (1927, p. 24). Their peculiarity consists in the onset of a thinning or lytic action spreading in from the margin on one side, and in their defective viability. They are very difficult to subculture, and may be only viable on agar and not in broth, suggesting the presence of bacteriophage. Arkwright (1921, 1924) obtained colonies perhaps similar to these from old cultures of *B. dysenteriæ* (Shiga).

Secondary colonies or papillæ.

Secondary colonies or papillæ produced on primary colonies are of special importance in regard to varying fermentation characters, and in this connection will be dealt with in a later section. Papillæ appear to arise as the result of certain bacteria at a given point in the colony developing variant characters which help them to grow in spite of the inhibiting effect exercised by the products of growth accumulated in the surrounding parts of the colony, or which actually enable them to make use of nutrient material unsuited to the wants of the parent strain. Papillæ usually indicate the appearance of a variant.

Sectors.

Variants may appear as altered or excessive growth in one sector of a colony which is several days old. These have been described by Baerthlein (1912) and Stewart (1926, 1927). Very striking instances have also been recorded by Nirula (1928) who worked with strains isolated by single cell methods from cultures of bacteria found in diseased plants.

Special Instances: Variant colonies of C. diphtheriæ (see Figs. 5 & 6) *and smooth and woolly colonies of certain anaerobes.*

Variants of C. diphtheriæ. A notable example of a variant which can be isolated by selection of colonies is the small, thin, granular type of colony of *C. diphtheriæ*. This was first described by Cobbett and Phillips (1897), and later with more detail by Slawyk and Manicatide (1898). It has been described by Bernhardt (1915), Bernhardt and Paneth (1913) and Baerthlein (1918). It differs from the larger, and, in some strains commoner, type of colony not only by its very irregular outline and uneven surface, but by the scanty and very granular growth in broth.

It is doubtful, however, whether this variant has much other analogy to the R variants of the *B. coli* group than the manner of growth on agar and in broth. Both forms of *C. diphtheriæ* cultures, but especially the granular form, make very unstable emulsions. Since they are almost equally unstable in distilled water and salt solution, it is difficult to say whether the presence of electrolytes has a similar effect on them and on a ' rough ' *B. typhosus*. Both types of agar growth of *C. diphtheriæ* retain their respective characters if subcultures are made at intervals of 7 to 10 days.

Old colonies of the thin granular form often produce on their surfaces secondary colonies or papillæ. These may grow so large as to cover the original colony, and then subcultures yield the larger, thicker type of colony which has a comparative freedom from coarse granules. Both these forms produce typical diphtheria toxin in broth cultures, and emulsions of the bacilli are pathogenic for guinea-pigs. Another variant has been studied by Cowan (1927), who obtained it from several distinct strains of virulent *C. diphtheriæ*. It could be recognized as a very small dense form of colony which produced very scanty growth in broth. No toxin could be detected in broth cultures, nor did large doses of the bacilli prove pathogenic for guinea-pigs either subcutaneously or intradermally. This form is probably identical with one of the variants described by Bernhardt and Paneth (1913).

Nungester (1927, 1929) describes seven variants of *B. anthracis*. His main types are the 'usual' or medusoid, which he calls 'R', the round and smooth or 'S' form, mucoid variants which may be 'R' or 'S', and phantom colonies. His variants have considerable resemblance to those of *B. subtilis* described by Soule. Nungester found all variants sporogenic, none motile and that there was no evidence of a 'cycle'. The degree of virulence was definitely related to the type of colony.

Smooth and woolly colonies. The two types of colony isolated by Schattenfroh and Grassberger (1900) from cultures of Fränkel's bacillus, *B. sporogenes* or *B. chauvœi*, which varied greatly in their appearance and biochemical properties and were regarded by them as variants, probably consisted of two distinct species, *B. perfringens* (or Fränkel's bacillus), and *B. sporogenes*. The experience of workers with these anaerobic species illustrates the great difficulty sometimes found in isolating pure cultures, even by the most laborious techniques (see Volume III).

Variation in Physiological Characters.

Very numerous variants distinguished by their chemical or other functions have been recorded, and variations have been observed in one species or another affecting almost every physiological character which has been studied. As in the case of the morphology of individual bacteria or their colonies, many of the variations, though to a certain extent hereditary, are apt, in the course of subculture, to become again approximated to the type by reversion. The variants can often be obtained fairly pure by the selection of single colonies which contain the desired

character in a marked degree. Unless, however, the new character is indicated by a distinctive appearance of the colony bearing the difference, the number of colonies which must be picked off for the purpose may be very large before one pure in type is obtained. Eventual success is a matter of chance unless the variant colonies are present in a large proportion, or have some distinctive appearance. Success in obtaining a pure variant is, therefore, to a large extent dependent on some peculiarity in appearance of the colony, which acts as an indicator. All the numerous kinds of variants in respect of physiological activities cannot be mentioned in this chapter, but a few instances will be given.

It may be noted that the difficulty encountered in obtaining pure variants by selection of colonies, when these do not show evidence of the change in their outward aspect, is very much greater if an attempt is made to select single bacteria under the microscope. By this latter technique, the search and successful selection is often made impossible, not only by the absence of an indicator, but by the very low viability of single cells.

Oxygen and CO_2 requirements.

It has been observed in the case of certain species that cultures when first isolated from the animal body will not grow when exposed to the air, but only below the surface in agar tubes ; after a number of subcultures growth can be obtained on the surface. This is the case with *Br. abortus* (Smith, 1924) and *B. acnei* (Molesworth, 1910), and to some extent with *Gonococcus* (Wherry and Oliver, 1916) and *Meningococcus* (Cohen and Fleming, 1918). The initial difficulty in growing *Br. abortus* is overcome by sealing the culture tubes, or more effectually, by exposing the culture to an atmosphere containing 10 per cent. of CO_2. The primary growths of *Meningococcus* and *Gonococcus* are also favoured by the presence of 10 per cent. of CO_2 in the air. Later changes in the culture occur which enable surface aerobic growth to take place without these special precautions. Whether this mass variation in the requirements of the micro-organism is the result of selection of favoured individuals is unknown.

There is no definite evidence of a strict anaerobe producing truly aerobic descendants. Organisms which appeared to be strict aerobes when first isolated sometimes become more tolerant of relatively anaerobic conditions later, e.g. *Pneumococcus, B. influenzæ.* Such changes in habit and requirements have not been thoroughly worked out by selecting and testing single colonies, nor have the specially required conditions been investigated in detail.

Modifications also occur of some other special requirements connected with change from parasitic and saprophytic life. Some bacteria when first isolated need serum, hæmoglobin, or special accessory factors in the medium, which can be dispensed with later. Among these are the *Gonococcus, B. influenzæ, Pneumococcus, Streptococcus, B. pertussis*, &c.

In the case of the last, adaptation to ordinary nutrient agar was shown by Bordet and Gengou (1911) to be associated with great changes in the morphology of the individual bacteria, and of the colonies, and also in the serological reactions.

In these and similar instances the changed needs can generally be explained by a survival of those individual bacteria present in the original culture which are best suited to the new conditions of growth and a more or less gradual production of an adapted race by selection. This method of change is, however, presumed from analogy rather than demonstrated, and, as in some of the other changes in bacterial cultures, a more direct influence of the culture media may also have its effect.

Fermentation of carbohydrates.

The production of gas from glucose was found by Smith (1893, 1895) to be a very constant and useful test for distinguishing between *B. coli* and *B. typhosus*. Later, the production of acid or acid and gas in many kinds of media and alcohols containing carbohydrates has been used as a means of differentiation. In spite of the usually constant behaviour of different species of bacteria in producing acid or acid and gas from such media, nevertheless, many variations in these respects have been noted. Neisser (1906) and Massini (1907) described *B. coli mutabile*, of which the chief peculiarity is that on such media as neutral-red-lactose-agar two kinds of colony appear, which respectively do and do not produce acid from lactose; the white colonies, if picked off to broth and plated again, produce both red and white colonies, whereas all the red colonies continue to produce only lactose fermenters. The apparent suddenness and very definite character of the change, signifying the appearance of a new function, induced Massini (1907) and Müller (1908) to regard the change as a distinct 'mutation'. Later observers have, however, sometimes noted colonies of intermediate character. The appearance of the lactose-fermenting individuals may also be manifested by the late production of acid in lactose-peptone-water, i.e. after 2, 3 or more days' incubation, or by the appearance of red papillæ on the white colonies on a MacConkey agar plate.

Very similar phenomena to those shown by *B. coli mutabile* have been described with regard to other carbohydrates in the cultures of other coliform bacteria. The late fermentation of cane sugar in peptone-water by *B. inconstans* was described by Braun and Loewenstein (1923) with a similar production of red and white colonies on cane-sugar-neutral-red-agar or of red papillæ on white colonies on this medium. The formation of red papillæ on white colonies was also recorded by R. Müller (1908) as a distinctive mark of *B. paratyphosus B* as opposed to *B. ærtrycke*, when these organisms were grown on raffinose-neutral-red-agar. Ledingham (1918) also observed the appearance of non-fermenting variants as papillæ when a strain of mannitol-fermenting *B. dysenteriæ* was grown on neutral-red-isodulcitol-agar.

Penfold (1911[1]) studied this phenomenon in detail in relation to the fermentation of dulcitol by *B. typhosus*. It was noticed that of several strains of *B. typhosus* inoculated into litmus-dulcitol-peptone-water, some formed acid in 1 or 2 days, but most did not till the fourth or fifth day or even later. If the culture was plated out daily on agar containing dulcitol and neutral red, a few red colonies sometimes appeared on the second day followed by an increase in the proportion of red to white colonies till about the fifth day ; when the peptone-water culture had become acid, all or nearly all were red. A subculture of the dulcitol-peptone-water at this time into the same liquid medium became acid in 24 hours. This rapid fermentation was, no doubt, the effect of the large number of dulcitol-fermenting bacteria present, as was shown by plating out. The gradual change in the properties of the culture was due to the occurrence at first of a few individual bacteria with some power of fermenting this alcohol. These individuals were then favoured and selected by the medium, which afforded additional nutriment to those bacteria able to ferment dulcitol. They alone were able to use the dulcitol as food, and so grew and multiplied in excess of the others. The white colonies of the original culture on dulcitol-neutral-red-agar when three days old produced red papillæ or secondary colonies on their surface, which were composed almost entirely of rapid dulcitol-fermenters, capable of forming red colonies when transferred to a fresh plate. The history of this variation in dulcitol culture media described with much very interesting detail by Penfold (1911[1]) represents a typical case of the transformation of a culture which is unable to ferment a certain sugar or alcohol into one capable of rapidly fermenting it.

The fermenting culture in the absence of further selection, e.g. when grown in ordinary broth, may again become, by reversion, a non-fermenting culture or one acting very slowly. This reverse change from the ability to ferment a sugar to a condition of inactivity was also studied by Penfold (1910) in his work on the Twort's lactose-fermenting strain of *B. typhosus*. Twort (1907), besides ' training ' *B. typhosus* to produce acid from lactose, described a number of similar variations in sugar fermentation, which he noted in cultures grown for a shorter or longer time in different sugars. The effect of prolonged growth was shown to be in the direction of producing variants which fermented the particular carbohydrates with which the strain was cultured. The process of change or variation was called ' training ', but Penfold's work suggests that the action of the sugar was largely selective and not due simply to a direct stimulus to a latent function. Penfold also studied the process of reversion of Twort's lactose-fermenting *B. typhosus* to the non-fermenting type. The readiness with which distinct changes, which are by no means rare, in fermentation characters take place in some strains of bacteria, and the variety of substances which are affected by these different variations, has raised the question of the permanence of fermentation characters and their value for classification. It is only when the constancy of certain physiological

properties in special cases is established by numerous observations, that their phylogenetic importance can be accepted, and much value cannot be assigned to such characters when they have only been observed in a few instances or during a short interval. Reiner Müller (1911) and Penfold (1912), however, considered that not only the existing fermentation characters of a culture but also its tendencies, i.e. the directions in which it could vary, should be taken into account in classification. This idea is in accordance with the view that a species of bacteria may exist in several different phases or so-called ' cyclostages '.

Gas formation. The formation of gas from sugars is a stage or completion of their hydrolysis, and has often been regarded as a very fixed and constant character ; it has indeed been used to separate large groups of bacteria, e.g. *B. coli* from *B. typhosus* and the dysentery bacilli. The absence of gas formation is an invariable feature in the hydrolysis of carbohydrates by many micro-organisms, e.g. *Staphylococcus*, *Streptococcus*, *Corynebacteria*, &c. Defective gas formation is also one of the variations which have been most carefully observed, and has been found to occur in several kinds of bacteria, normally producing gas, of the intestinal Gram-negative group. Revis (1911, 1912) found that *B. coli* grown on 0·05 per cent. malachite green might lose its ability to form gas from alcohols and sugars, and Penfold (1911[2], 1912) showed that growth on chloracetic acid agar had a similar effect in the case of several organisms such as *B. coli*, *B. enteritidis*, &c. He showed that the inability to form gas depended on failure in the power to split formic acid. Formic acid could be acted on with the formation of gas by gas-producing bacteria, but if formic acid was not produced, or if, as in the case of *B. typhosus* or *B. dysenteriæ*, the organism was unable to split formic acid, then gas did not appear. For these reasons a mixture of *B. typhosus* and *B. morgani* produced gas from mannitol though neither alone could do this. *B. typhosus* made formic acid from the mannitol and the other organism made gas from the formic acid. Sears and Putnam (1923) further investigated this phenomenon, and Castellani (1925, 1927) has recorded similar observations.

The loss of the aerogenic function was persistently hereditary, but eventually reversion might take place under conditions which Penfold studied in detail. The reversion, as well as the original change, occurred first only in a few individuals of a culture. Reversion occurred more rapidly in a large quantity of liquid culture medium. In a 2- or 5-litre flask, the change occurred more readily than in 5 or 10 c.cm. This fact Penfold attributed to the greater chance of the needed variant occurring among the larger number of bacteria present in a large flask.

Changes in the fermentation characters of other kinds of bacteria, e.g. streptococci (Ainley Walker, 1910), staphylococci and meningococci, undoubtedly do occur, though many writers have pointed out that under uniform conditions these characters may be extraordinarily constant in the same strain over long periods of time (Gordon, 1905, 1911). These

observations chiefly relate to growth in liquid media of mass cultures which were originally purified by the selection of single colonies. It seems probable that some of the variability described has been due to impurity of the original culture, an accident which is especially likely to occur in dealing with the minute colonies of the streptococcus, a class of organism comprising many species and races which occur together and are very similar in morphology. This impurity of colonies is especially liable to occur if it is difficult to obtain a well emulsified inoculum for the agar plates. In order to detect variation proceeding in a culture it may be necessary to examine a large number of separate colonies, since the variants will often be few in number in the colonies, and will consequently be unlikely to produce obvious changes in a mass subculture.

Variants have been described which exhibit very striking difference from the parent in the amount or concentration of acid produced in cultures. Gorini (1921) obtained a variation in milk cultures of a spore-forming bacillus, *B. acidificans*. The variant produced much less acid, and did not clot the milk like the parent culture, while the casein was digested by both forms. In (1928) he described two races obtained from a culture of the same bacillus which grew in colonies of different form (smooth and irregular, respectively), and were distinguished also by their different abilities to produce acid and clotting.

Decomposition of proteins.

The chief variations observed under this heading are those concerned with the liquefaction of gelatin, inspissated serum or milk clot and with putrefaction. The production of indole is sometimes a variable character, as has been especially noted in the groups of dysentery bacilli and *B. influenzæ*. Variants have been described in Salmonella, which, unlike the parent and other members of the group, produced indole ; these latter variants are not generally accepted as proved, but v. Loghem (1919) has recorded in detail the history of a strain of *B. paratyphosus B*, some variants of which produced indole after it had been subcultured for a long time. In some cases indole produced early in a culture is destroyed later and this may give an undue appearance of variability, as in the case of *B. inconstans* (Braun and Loewenstein, 1923). The liquefaction of gelatin by the *V. choleræ* (Baerthlein, 1912) and by *B. proteus* or staphylococcus, is subject to great variation. Variants lacking this character have been obtained from strains in which the parent cultures had the faculty well developed. The inability of old laboratory cultures of *Staphylococcus aureus* to liquefy gelatin is another well-known instance. Variations in the power to liquefy inspissated serum is less well authenticated and the alleged variation of *B. sporogenes* in this respect, described by Schattenfroh and Grassberger (1900) is usually attributed to the impurity of the original culture, since the complete separation of the cultures of this bacillus from *B. welchii* is known to be often a very difficult task. v. Loghem (1911) investigated the variable

ability of strains of *B. typhosus* to use the nitrogen in ammonia for growth, and recorded changes in cultures in this respect.

Pigment formation.

Many observers, Dudgeon (1908), Bigger, Boland and O'Meara (1927), and others, have noticed the occurrence of white and orange colonies on agar plates of *Staphylococcus aureus*. Cultures from the white colonies usually retain their colourless character, but pigmented colonies are less constant. Variants of *B. prodigiosus* have also been studied by plating and selecting colonies of special colour. In this way cultures which were white, pink, red and purple, have been obtained of variable degrees of persistence (Rettger and Sherrick, 1911). Strains of *B. pyocyaneus* with well developed or absent power to form the characteristic blue pigment can be obtained in the same way (Gessard, 1919).

Hæmolysis, &c.

Two distinct metabolic functions have been included under this heading : (1) the secretion of an enzyme in broth cultures which lyses red blood corpuscles. Loss of this character has been assumed sometimes in cases where it has merely become less readily demonstrated ; for instance, Lyall (1914) and Cowan (1922) found that the centrifugate from a broth culture of *Streptococcus hæmolyticus* did not lyse sheep's red blood corpuscles because the cocci had grown at the bottom of the tube, but that this character was readily demonstrated if the cultures were well shaken up before they were centrifuged to obtain the hæmolysin. Unless this was done the lysin formed by the streptococci was deposited and not distributed throughout the liquid. There is, however, no doubt that the hæmolytic power can be lost in variants. It has also been found that some streptococcus strains lyse rabbit's red blood corpuscles, but not those from man, and this character has been found to be variable. The hæmolytic power of some cultures of *V. choleræ* has also been shown to vary : one variant forming clear colonies which did not cause hæmolysis and another with yellow colonies causing hæmolysis were described by Baerthlein (1912).

(2) The bleaching of the hæmoglobin and the rendering transparent of the area immediately surrounding a colony on blood agar has also been termed hæmolysis, but appears not to depend on the same function. v. Loghem (1913) described the two functions as hæmolysis and hæmodigestion. The latter phenomenon may occur on blood agar when no hæmolysin can be demonstrated by means of broth cultures. Individual colonies may show defect in this respect and the change may be hereditary. This variation also was demonstrated by Baerthlein (1912) in the case of *V. choleræ*.

Solubility in bile-salt.

This has been taken by many workers, especially of late years, as the character of pneumococcus which most surely differentiates it from streptococci. Morgenroth, Schnitzer and Berger (1925) found that the solubility varied after the culture had been submitted to the action of

certain drugs, especially optochin. The variants which resisted the action of bile-salt had also lost their virulence and were devoid of capsule. Similar changes were described as characteristic of 'degraded' or R forms of pneumococcus by Reimann (1925). Atkin (1926) described variation in the autolytic capacity of pneumococcus, which function he believed to be concerned in its solution in the presence of bile, and to be merely accelerated by the latter reagent.

Agglutination by acids.

Sobernheim and Seligmann (1910) and Seligmann (1926, 1927) described changes in the serum agglutination of certain strains of *B. paratyphosus B*, which, in the variant form approached *B. enteritidis*; and Beniasch (1912) found that the variants of the same paratyphoid bacillus also became assimilated to *B. enteritidis* as regards acid agglutination. The variation described by these authors may now readily be explained by the difference in agglutination reaction, both as regards specific sera and acid, which are known to be associated with the H and O, and S and R forms, and the specific and non-specific phases described by Andrewes (1922) (see Arkwright, 1928).

Agglutination by neutral salts.

Emulsions of many bacteria are quite stable in a solution of NaCl 0·85 or even 1·4 per cent., but others, e.g. many strains of *C. diphtheriæ*, *Staphylococcus*, *Streptococcus*, &c., are clumped and precipitated, or are only very imperfectly stable, both in distilled water and physiological salt solution. Others which are stable in distilled water, are not, in neutral salt solutions such as 0·85 or 0·42 per cent. NaCl. It has long been known that some strains of *B. coli*, *B. typhosus*, *B. dysenteriæ* and other members of this group (Nicolle, 1898; Savage, 1901; v. Lingelsheim, 1913; Arkwright, 1914, 1920, 1921) become 'spontaneously agglutinable', i.e. tend to form clumps in salt solution in the course of laboratory culture, and it was shown by Arkwright (1920, 1921) that certain variants which appeared on agar plates as granular, irregular, opaque or 'rough' surfaced colonies, were readily emulsified in distilled water, but agglutinated if the NaCl content was raised above 0·4 per cent., or sometimes even when higher than 0·1 per cent., and that such variants formed a large deposit in broth with clear supernatant. That the deposition was due to clumping on account of the high salt content was shown by the fact that the same variant made a diffuse turbid growth in broth with low concentration of salt, but was precipitated after the addition of more salt. This character constitutes one of the most readily observed differential characters of the rough or R form in the *B. typhosus-coli* group.

Though the character of salt-agglutinability usually distinguishes the R form from the normal or smooth form, the most essential difference resides in the different constitution of the S and R somatic antigens. The spontaneous agglutinability of the R variant may sometimes be lost, so that emulsions which are stable in salt solution can be made, and broth

cultures may grow with uniform turbidity without the essential serological character of the agglutinable substance having reverted to the S type. White (1927, 1928) has shown that R cultures can be made to emulsify uniformly in salt solution if the bacilli are extracted with alcohol, and he believes that the stability of some R cultures and of extracted R bacilli is due to the same cause, i.e. the loss of a lipoid substance, without any change taking place in the R somatic antigen. He has also pointed out that R emulsions often to some extent agglutinate with normal serum. He considers that the 'spontaneous' agglutination is of four kinds, (1) due to the loss in the R variants of a 'specific soluble substance' which is present in the S antigen and maintains the dispersion of the emulsion, (2) due to excess of lipoid which is characteristic of some cultures and promotes clumping without diminution of the normal amount of specific soluble substance, (3) agglutination of some R cultures after heating, due to denaturation of the protein of the somatic antigen, (4) in the presence of normal serum due to its reaction with the lipoids of the bacilli. The agglutination of R emulsions by neutral salts is due to the kation, and is connected with the charge on these ions. It is accordingly brought about by much weaker solutions of salts with bivalent (calcium or magnesium) or trivalent kations than by those with monovalent kations (e.g. sodium or potassium).

Resistance to heat, &c.

Strains of bacteria adapted to unusual temperatures have been obtained by training, e.g. by keeping cultures at raised temperatures and sub-culturing frequently or by raising the temperature gradually. Well-known instances of this are to be found in the case of mammalian *B. tuberculosis* accustomed to grow at 40° C., and of *B. anthracis* to 40° C. In the latter instance, virulence is impaired and this method was used by Pasteur to prepare his attenuated anthrax vaccine. Strains pathogenic for birds and mammals have also been accustomed to grow at lower temperatures, e.g. 20° C., although when first isolated requiring a higher temperature for growth.

Some strains of bacteria have also been trained to resist phenol and other chemical substances, and the growth in broth or on agar containing as much as 0·1 per cent. phenol has been used to eliminate the heat-labile antigen from normal *B. proteus, B. typhosus* and *B. enteritidis* cultures (Weil and Felix, Braun and Schaeffer, Goyle). The change in this instance has generally been gradual and not persistent, relapses occurring in one or two subcultures after a return to ordinary conditions.

In a similar way bacteria have been trained to resist other chemical substances and in several instances correlated changes have been noticed; for instance, the loss of the faculty of producing gas from certain alcohols and sugars by growth with malachite green by Revis (1911), or in chloracetic acid by Penfold (1911[2]). Morgenroth, Schnitzler and Berger (1925) obtained strains of pneumococcus resistant to optochin, rivanol, &c.,

and described associated changes in virulence and other characters. Marks (1910), extending the earlier observations of Danysz (1900) and others, recorded the adaptation of a strain of *B. paratyphosus* till it would grow in a 1 in 3,500 solution of arsenious acid, and exhibited altered serological properties.

Sensitiveness to bacteriophage.

It is well known that after the action of bacteriophage on a young culture of *B. dysenteriæ* or *B. typhosus*, &c., growth occurs which is resistant to that particular phage, and that this resistance may persist although the culture has again become apparently free from phage (see Gratia, 1921 ; d'Herelle, 1922[2], &c.). Similar and independent resistant states apparently may also be developed to different strains of phage simultaneously. Arkwright (1924), working with a single strain of phage, found that it acted on R variants of *B. dysenteriæ* (Shiga) and not on the S form, though all R forms were not susceptible. This exclusive action on R variants was shown to apply to all of nine strains examined. Hadley (1926) described similar phenomena, and Burnet (1927) has gone further, and shown that strains of phage can be obtained which only act on the S form of bacteria, and others which only act on the R forms, and that these latter have a wider range of activity, corresponding to the wider range of affinity of the R antigen. The resistant colonies derived from the S form by bacteriophage are R, and appear to owe this change directly to the action of the phage. To some extent at least the reverse is true, i.e. the resistant variants resulting from the action on the R are found to be S forms, but in the latter case the evidence was not sufficient to show whether the resistant S colonies were mere survivals from a mixed growth which had come into prominence by destruction of the R individuals. D'Herelle attributes much, if not all, bacterial variation, especially the transition from S to R, to infection of the bacteria with bacteriophage, although the presence of the phage may be impossible to demonstrate directly. Mellon (1926) and Hadley (1927) also appear to hold the view that bacteriophage is one of the most important but often unnoticed causes of variation. Bordet (1923), Gratia (1921) and Arkwright (1924) have pointed out the very striking effect of phage in producing variation, but rather look on this action as an intensification or modification of a normal process of metabolism of which phage action is an extreme case.

Variation of Characters Related to the Parasitic Life and Activities of the Micro-organisms Concerned.

Of these changes the earliest noticed and most frequently recorded has been the decline in ' virulence ' of strains of pathogenic bacteria after isolation from the animal body. In this connection virulence has been generally used to denote pathogenicity in general without analysis of this form of activity into invasive power, ability to grow, multiply and survive in the body, reaction to lytic action of the serum or to phagocytosis, &c.

The ability to form toxin has also been observed to vary considerably in the same strain. The virulence, strictly speaking, has been chiefly investigated in the case of streptococci, pneumococci, *B. pestis*, *V. choleræ*, and *B. anthracis*, and toxigenicity in *C. diphtheriæ*. Variability in the power of producing toxin at different times by the same strain of *B. tetani*, or of other toxigenic anaerobes, has been to some extent studied, though observations have chiefly been made on the different toxigenicity of strains isolated from different sources.

Virulence.

The decline in virulence in laboratory strains cultivated on artificial media has been a great obstacle to experimental work. The virulence of freshly isolated strains has been to some extent correlated with resistance to bacteriolysins and phagocytosis. Formerly, observations were usually made on the change affecting the culture as a whole, and no attempt was made to test the uniformity of the change throughout the culture. Recently, differences in virulence have been found to be associated with definite forms and variants, sometimes recognizable by the appearance of the colonies. In the pneumococcus, Griffith (1923) showed that the colonies of non-virulent variants might be granular and distinct in appearance, and were readily obtained by growth in the serum of rabbits immunized to the same type of pneumococcus. The cultures in broth were turbid and only slightly granular and emulsions in salt solution were stable. He called this variant ' rough ' and believed that it was a parallel form to the rough *B. dysenteriæ*, &c. Heidelberger and Avery (1923, 1924) associated the change with loss of capsule and of the ' soluble specific substance ' characteristic of each type (cf. Reimann, 1925–6). The change in virulence in the case of Griffith's cultures was from a m.l.d. dose for mice of one-millionth of a c.cm. to more than 0·1 c.cm. Cowan (1922, 1923, 1924) had previously found considerable differences in the virulence of cultures made from ' smooth ' and ' rough ' colonies of the same strain of hæmolytic streptococcus. These non-virulent colonies were found by plating out old broth cultures on agar. The non-virulent variant grew in broth in long chains, and was deposited, leaving the broth clear. Cowan's observations were carried out with a number of different strains, which were tested on rabbits.

Todd (1927) and Andrewes (1928) also working with hæmolytic streptococci have found that the colonies which have the most irregular outline and consistency, and which also grow as tangles and deposit in broth, are more virulent than the ' smooth-looking ' colonies of uniform consistency. The discrepancy has not yet been finally explained, but Eagles (1928) has found that with some strains there is a lowered virulence associated with the ' rough ' form such as was found by Cowan, but that when he examined a very virulent strain (' Aronson '), the irregular colonies associated with granular broth deposit were far more virulent than the growth from those colonies which were ' smooth '. It seems

probable that in very many cases the irregular aspect of the colonies and the granular or clear broth cultures merely denote the growth of the streptococci in long chains, and that this characteristic, though sometimes associated with agglutinability by salt, is by no means always an indication of virulence or the reverse, but is to some extent a sign of other differences in the individuals giving rise to the colonies, and in this way may prove a valuable hint in searching for variants. The correlation of irregularity or of smoothness of colony with virulence, however, is not constant.

Observations on the variation in virulence of strains of *B. anthracis* have been made by a great many workers since the classical experiments of Pasteur, and his use of a living non-virulent strain of this bacillus for prophylactic vaccination. Considerable evidence has been brought forward suggesting that the production of a capsule is correlated with virulence and more especially with resistance to phagocytosis (Preisz, 1911). The direct dependence of virulence on the possession of a capsule in anthrax is not, however, universally accepted (Fischoeder, 1909). The work of Heidelberger and Avery (1923), Reimann (1925) and others, on pneumococcus, brings strong evidence that in this species, at any rate, virulence is very closely connected with capsulation and the chemical components of the capsule, and may lead to reconsideration of the evidence for connecting the capsule and virulence in other species of bacteria also. The virulence of the pneumobacillus of Friedlander also has been shown to be closely associated with the development of the capsule (Toenniessen, 1921; Reimann, 1925; Julianelle, 1926, 1928) and corresponding characters of the colonies.

Attenuated strains of many kinds of pathogenic bacteria have been sought for to use as living vaccines. Very few permanently non-virulent strains suitable for this purpose have been found. Calmette (1922), and Calmette, Boquet and Negret (1921) have obtained a strain of *B. tuberculosis*, ' B.C.G.', which originated from a virulent strain grown in bile. It is believed by them to be quite non-virulent and has been used as a living vaccine for calves and children. The complete permanence of its innocuous character has been questioned by Watson (1927) and McIntosh and Konst (1928). Both Pasteur's attenuated *B. anthracis* and ' B.C.G.' are examples of persistently non-virulent strains obtained by growth of mass cultures under special conditions, and not by the artificial selection of variant bacilli or colonies. Nungester (1929) found that the different variants of *B. anthracis*, which he recognized by the form of their colonies, were of different degrees of virulence.

Variation in the opposite direction, i.e. from low to high virulence, has usually proved very hard to induce in artificial culture, but passage through the animal body has been used successfully in many instances, e.g. streptococci, pneumococci and *B. pestis*, &c. The resuscitation of virulence by this means has generally been attributed to the selective action of the body fluids and cells, which kill off or restrain the non-virulent forms, and only allow the survival and propagation of the virulent.

Passage through living hosts as a means of producing variants of any kind is not a very satisfactory method of extending knowledge on variation, since besides the difficulty of ensuring that the cultures recovered from the body are descended from the inoculum, the complicated processes of action and reaction between the bacterium and the host frequently completely baffle analysis. The experiments of Griffith (1928) with the pneumococcus, must, however, be mentioned. This observer succeeded in not only obtaining a capsulated virulent pneumococcus from a non-capsulated non-virulent culture by passage through a mouse, introducing a killed culture of virulent capsulated pneumococcus subcutaneously at the same time, but also in changing the *type* of living culture inoculated, so that the new virulent culture derived from the blood of the mouse after death was of the same type as the dead culture used. The non-virulent culture descended from a type I, for instance, had become changed into a virulent type II by association in the tissues of the mouse with virulent type II culture killed by steaming. These results, which suggest new and very important processes concerned with variation of bacteria, were carefully controlled and repeated. They have since been confirmed by Neufeld and Levinthal (1928).

Passage of cultures through serum *in vitro* has sometimes been found an effective method of raising virulence. The result has usually been attributed to a selective action of the serum by destruction or inhibition of the less virulent members of the culture. This explanation is by no means entirely satisfactory, and a harmful or attenuating action of the serum can often not be demonstrated; moreover, the serum may be quite effectual although it has been heated at 56° C. for half an hour. Growth for 24 hours in broth with the addition of 10 per cent. heated horse-serum was found by Rowland (1914) greatly to increase the virulence of *B. pestis* for rats. The rapidity with which increased virulence is produced in some such instances and again disappears, suggests that the variation is possibly of a temporary nature due to the nutrition of the bacteria with a particular pabulum, rather than to a true hereditary variation. This question requires further work with single colonies before a satisfactory answer can be obtained.

At one time it was believed that growth in immune serum might still further enhance the virulence of cultures by subjecting them to a more stringent selection by specific lysins (Trommsdorff, 1901; Pfeiffer, 1902; Hamburger, 1903, and others). The reverse action of immune serum has of late been demonstrated in certain cases, e.g. pneumococcus (Griffith, 1923; Reimann, 1925). The effect of growth in specific antiserum of these bacteria, as well as other species (*B. typhosus*), is to induce or accelerate the change from the smooth form to the rough with accompanying loss of virulence. The diminished virulence which has been recorded as the result of passage through the body of an immune animal has been dealt with by Morgenroth and others (1925) under the head of 'depression immunity', and the same writer

published evidence of similar loss of virulence as the result of the action on pneumococci of certain drugs, e.g. optochin and rivanol.

The numerous devices recommended for maintaining or restoring virulence to cultures testify to the readiness with which this property is lost in artificial cultures. It seems clear that growth in ordinary laboratory culture media, but especially such as contain immune serum, or passage through an immune animal, tends to depress the virulence of a strain, and that growth in normal serum or in a susceptible animal often raises virulence. This will be again referred to in the section dealing with the different kinds of antigens in bacteria and their relation to virulence.

It seems probable that in the case of virulence acquired by passage through a susceptible animal or through culture medium containing serum, the bacteria take up something into their composition which is necessary for their virulent activity. If the virulence is then truly hereditary, this must imply that a working mechanism capable of forming anew the required component is set up in the bacteria under the influence of the material taken up from the medium. In some cases the component which conditions virulence is probably present in great excess, and may, perhaps, be taken up and stored so as to supply a large number of future generations of bacteria, without true biological inheritance occurring. The essential material, whether food, vitamin, catalyst or enzyme, may be passed on in minute quantities from generation to generation, or even taken up by several new generations of living bacteria from the dead members of the culture.

Adaptation.

A character particularly subject to variation and allied to simple virulence but not always identical with it, is that known as adaptation of a pathogenic organism to a particular host. Thus, a streptococcus may be highly pathogenic for the mouse but not for the rabbit and *vice versa*. A similar lack of adaptation may be found in strains of anthrax which are virulent for one but not for another host. The same facts have also often been observed in the case of *Pasteurella*. One of the most remarkable instances of this phenomenon is found in the ultramicroscopic viruses. Thus, smallpox virus can with great difficulty be adapted to the calf and only rather more readily to the rabbit. A strain of foot-and-mouth virus which is highly virulent for the ox may require several passages through guinea-pigs before its virulence for this animal has attained its maximum. Similar experience is met with in adaptation of this virus to rabbits.

In the case of adaptation to a new host a change in the bacteria may take place similar to that associated with increase of virulence. Some special constituent of the animal body in question may be taken up by the bacterium and act as a special means of promoting contact between the parasite and host, and may thus assist the attack on

the animal cells. Such a hypothetical adjuvant may be compared to a flux which is needed in bringing about a simpler physical union.

Toxigenicity.

The different value of toxin produced by different strains of *C. diphtheriæ* is notorious. Changes in the capacity of a single strain for production of exotoxins are also commonly observed in laboratories devoted to the manufacture of antitoxins. This uncertainty has often been attributed to slight differences in the culture medium in use, and, no doubt, exactly prepared appropriate media are of great importance. In other cases there seems to be no doubt that the low grade of toxin production is due to variation of the bacterium (Cowan, 1927). The new form present may be a variant which makes less growth than a more serviceable strain, or may be less toxigenic though equally prolific. An example of this kind of variation is to be found in Baerthlein's (1912, 1918) variants of *C. diphtheriæ*, which produced little or no toxin, and the non-toxigenic variety obtained from highly toxigenic strains of *C. diphtheriæ* described by Crowell (1926) and Cowan (1927). In the last-mentioned instance the colonies of the non-toxic variant were very small and the growth in broth slow.

Antigenic properties.

When the variation in the biochemical functions of bacteria such as the production of acid and gas from carbohydrates was investigated by Twort (1907), Penfold (1911[1 & 2]), Arkwright (1913), and other workers, the proof that the variant obtained was really derived from the parent culture and was not a chance contaminant was afforded by the reaction of both parent and offspring with the same agglutinating serum. This criterion of the identity of a strain was rudely disturbed when it became certain from the work of Smith, Reagh, Orcutt, Weil, Felix, Arkwright, &c., that a strain might lose a part or all of its agglutinogenic and agglutinable properties and sometimes acquire new ones, and yet undoubtedly be descended from the original culture. Such an occurrence was not generally recognized as possible until within the last ten or twelve years. The state of opinion in 1912 is shown by Kolle's statement in Kolle and Wassermann's Handbuch, 2nd Edition, Vol. I, p. 899, that the specificity of which the immunity reactions are the foundation-stone is extraordinarily constant in spite of mutation in other respects. The same statement is made in the 3rd Edition, 1928, with qualifications as regards partial changes. This opinion was a natural one, seeing that experience proved that many cultures in favourable conditions changed remarkably little, and it was supported by the theoretical consideration that these reactions were dependent on the chemical nature of the proteins peculiar to the species, and that apparently analogous relations and differences between species of higher animals could be shown by precipitin reactions.

The earlier cases of variation in the antigenic characters were the so-called inagglutinable strains of *B. typhosus*, which had usually been

very recently isolated from the body, and especially from the blood or urine, and did not agglutinate like a normal strain with stock *B. typhosus* serum. These strains, which were said to be 'serum-fast', could, as a rule, be identified by the fact that when they were inoculated into rabbits, agglutinins for the normal type were produced, and often by the reappearance of agglutinability after the strain had been subcultured several times in broth. This phenomenon was explained by reference to Ehrlich's views of the compound nature of toxins and other antigens, as a case in which the agglutinogen had lost the toxophore or ergophore group but retained the haptophore, whereby it was able to stimulate the production of antibody though itself inagglutinable. The fact that in some of these cases the inagglutinable strain was non-motile has pointed to another interpretation. The non-motile culture having lost the greater part of its flagellar antigen, would no longer agglutinate rapidly in large loose conspicuous flocculi with a highly diluted serum like the normal form, but might retain sufficient to absorb a certain amount of agglutinin *in vitro*, and to stimulate agglutinin production in the rabbits. The records of inagglutinability of some strains recorded formerly do not appear to be fully explained in this way, but such occurrences have not been observed and fully investigated since the existence of separate somatic and flagellar antigens has been thoroughly appreciated. Felix (1922) and Felix and Olitzki (1928) record a high percentage (about 25 per cent.) of O (non-motile) strains amongst those isolated from the blood in cases of enteric fever, and they believe that their occurrence explains the recorded so-called inagglutinable strains. The absence or great reduction of flagella, and the flagellar antigen, which is the same as the H antigen of Weil and Felix, was described by these writers in the case of the O variant of *B. proteus X 19*, the strain which has a special relation to typhus fever, and is used for the agglutinating emulsion in the Weil-Felix reaction in that disease. From many species of motile bacteria besides the proteus group, especially in the enterica and Salmonella groups, the so-called O variants of Weil and Felix can be obtained. The O form of *B. proteus* was first distinguished by the fact that it grew in isolated colonies on agar in the absence of the spreading filmy growth or ' Hauch '. A culture so characterized was described as ' ohne Hauch ' (O) and distinguished by the absence of the H or heat-labile agglutinable substance and antigen which is associated with the presence of flagella. The O form may be temporarily produced by growth on 0·1 per cent. phenol agar (Braun and Schaeffer, 1919), in very weak broth or other inhibitory solid culture media. The H antigen or component can be destroyed in an emulsion by such means as heating at 100° C. for $\frac{1}{2}$ to 2 hours, or by the action of alcohol. The O form produced by culture on phenol, &c., however, is prone to revert to the normal, motile form. Pure, nearly or quite irreversible O strains have been obtained chiefly by repeated selection of colonies, and it is quite common for a completely non-motile O variant of *B. proteus* or *B. typhosus*, &c., to persist unchanged through many

subcultures. The fact that an apparently completely O variant may cause the production of H agglutinins when inoculated into a rabbit, is probably due to the antibodies being evoked by a comparatively small remnant of H antigen unrecognizable *in vitro*. It may also happen in such a case that there is sufficient H antigen left to absorb the H agglutinins, when large quantities are used for the absorption. White (1926, p. 39) found only a very small amount of H antigen was sufficient for demonstrating the absorption of H agglutinins *in vitro* and less still for stimulating the production of antibodies *in vivo*. The criticism which has been directed against the separate existence of H and O (flagellar and somatic) agglutinogens and agglutinins on account of such experiments is not very cogent.

The H or flagellar antigen.

Hereditary variation of the H or flagellar antigen which has been described so far has been of three kinds : (1) the reduction or complete loss of this component as in the O or non-motile form described above, or, on the other hand, its appearance in cultures hitherto believed to be non-motile ; (2) the loss of a part, as occurs in some members of the Salmonella group described by Schütze (1922), when a strain endowed with several H antigens loses one or more, and so changes from the main type to a subtype with fewer H antigenic components ; and (3) the change from specific to non-specific phase, or the reverse, of certain Salmonella strains described by Andrewes (1922). This change of phase concerns the H antigen, but the variation is often, though not always, of a very fugitive nature. It is possible that if sufficiently persistent attempts were made to procure permanent specific and non-specific phases these would be more frequently recorded. Scott (1926) described very persistent phases of this kind occurring under natural conditions, i.e. in strains recently isolated from the human subject. In this variation, as originally described, a culture which agglutinates with sera of two or more distinct types, e.g. *B. paratyphosus B* and *B. ærtrycke*, when plated on agar is found to form colonies which appear exactly alike on inspection, but when further examined without further subculture or after transference to agar or broth for a short time, have very divergent agglutinating properties. The one kind agglutinates almost exclusively with homologous serum, the other moderately or only slightly with homologous, but equally well or even to a higher titre with a number of sera made from other types of the same group. In a given culture the proportion of antigen which is 'specific' to that which is 'group' or non-specific, varies with the strain under examination and may change rapidly as the culture ages. In the same way the number of colonies which belong to the specific or group phase is also very different for different strains. It is generally agreed that in many strains even if colonies are examined with the least possible delay there is usually a considerable change-over at each plating, that intermediate colonies

are infrequent and that the segregation of specific and group phases is remarkably distinct and constant in quality.

The somatic antigen. Variants in which the heat-stable, somatic or O antigen has undergone a change such that it no longer reacts with the serum prepared from the normal or original form, are common in old laboratory cultures and can often be distinguished by the appearance of the colonies. Such variants are usually those known as the ' rough ' or R form. These forms and the accompanying antigenic changes have been described in several groups of bacteria, e.g. (1) *B. dysenteriæ*, *B. typhosus*, *B. coli*, Salmonella ; (2) *Pasteurella*, *B. lepisepticus* (De Kruif, 1921) ; (3) *Pneumococcus*.

A change of a similar kind from the S to the R somatic antigen has not been proved to occur in all groups of bacteria, but in many families an R form distinguishable by the appearance of the colonies has been described associated with a granular growth in broth, and in some cases with alteration in the reaction with agglutinating serum, and in the behaviour of the agglutinins obtained from rabbits. Loss of virulence has also been recorded as a characteristic of several R variants. The change in the somatic antigen often affects the manner of agglutination. Bacteria endowed with the S or normal somatic antigen, but with no H antigen, agglutinate with a serum prepared against the normal smooth culture in the form of compact small granules, like grains of sand, and large enough to be visible to the naked eye. When, however, the R is the only somatic antigen present without any H antigen, the bacteria do not agglutinate, or only in low dilutions, with serum made versus the whole smooth culture or with that versus the smooth non-motile O form, but do agglutinate with a serum prepared with an R form, whether it contains H antigen or not. The clumps then formed by the R emulsion are, as a rule, smaller than those formed by the smooth emulsions, and are usually too small to be seen separately with the naked eye, so that when shaken up the deposit appears to be muddy rather than sandy.

The statement that no agglutination of the non-motile R form occurs with S serum is not strictly correct, since the R form, especially in some cases, agglutinates with any normal or S serum, sometimes to such an extent as to render the test inconclusive. The converse test is more satisfactory, however. A serum made with the R form contains no agglutinins for the non-motile S form, so that the S and R somatic antigens can be shown to be distinct.

The somatic agglutinogen in the *proteus* and *typhosus-salmonella-dysenteriæ* group has been shown by Weil and Felix (1920), Balteanu (1926) and others, to be closely related to and probably identical with the greater part of the antigen which is concerned in complement fixation. The bactericidal and opsonic activities of an antiserum are probably also mainly concerned with the somatic antigen (Felix and Weil, 1920 ; Felix and Olitzki, 1926). It is accordingly found that when variations in the somatic agglutinins occur, the complement fixation, bactericidal and

opsonic reactions are altered in a parallel manner. It is, however, held by Braun and Nodake (1924), Balteanu (1926), Hofmeier (1926, 1927) and others, that the flagellar antigen (ectoplasmic receptor of Braun) and its antibodies are also involved to some extent in the complement fixation, bactericidal and phagocytic reactions.

It is not yet quite clear whether the S and R forms of Salmonella, *B. dysenteriæ*, &c., correspond strictly to those described in the *Pneumococcus* and *Pneumobacillus* of Friedländer. An actual chemical change in the antigen has been found in the R form of the *Pneumococcus* by Heidelberger and Avery (1923) and Reimann (1926), and also of the *Pneumobacillus* (Julianelle, 1926). This is believed to be due to the loss from the S antigen of a carbohydrate, the specific soluble substance, associated with the presence of the capsule : the remaining R antigen being apparently protein deprived of this non-protein fraction. The somatic R antigen may, therefore, be regarded as 'degraded' and this term has been used by Reimann to describe it, and the R form of *Pneumococcus*. Similar chemical alteration accompanies the change from the S to the R form in *B. typhosus* and Salmonella, according to Zinsser and Parker (1923), Landsteiner and Furth (1927), P. B. White (1926), &c. The carbohydrate fraction of antigen characteristic of the S form is called a 'hapten' by Landsteiner and Simms (1923) who have shown that such substances alone cannot excite the formation of antibodies, but only in combination with a protein. In considering the correspondence between the antigenic character of the R variant, as described above, and its more obvious cultural characteristics, the occurrence of two well-defined exceptional appearances which may lead to error must be mentioned, though they are more fully described in the previous section. These are (1) irregularity in the colony and precipitation in broth on account of the thread and chain formation in some cultures associated with normal or S somatic antigen ; (2) the turbidity of broth and smoothness of colonies of some cultures with R antigen.

Significance of the R form. The R variant is sometimes regarded as a degraded form, in support of which suggestion it is pointed out that it appears to have lost one of the important constituents (specific soluble substance) of the somatic antigen of the S form. The expression 'degenerate' or 'degraded' is perhaps not very well chosen, however. The idea is derived mainly from the fact that the S state is commonest in virulent cultures recently isolated from the animal body. Nevertheless, the R form often grows very vigorously in culture media and may be very long-lived in culture, since it is often the only survivor in old culture tubes. It is possible that a more correct view is that the R variant is the more saprophytic form, but that it acquires additional components and attributes during residence in the animal body and that these are correlated with virulence, but are soon lost in ordinary culture media. One aspect of this view has been elaborated by White (1926), who emphasizes the properties which are held in common by many R forms

derived from different species. He also suggests that a classification of the intestinal Gram-negative group may be based on the serological reactions of the R forms. In this form the serological relationships correspond more to families or ' genera ', and the distinctions are not fine enough to separate the smaller units or species, for which latter purpose the reactions of the S forms are needed.

The constitution of the somatic antigen of bacteria may also be altered without the occurrence of the R change, either by the number or character of its antigenic components varying. Thus, in the mannitol-fermenting strains of *B. dysenteriæ*, the serological properties may alter so that a smooth somatic antigen may disappear or be replaced by another, since in this group several somatic antigens can often be detected in one strain. In the case of *B. proteus X 19*, Felix (1922) obtained permanent variants of the O antigen, such that the O form no longer agglutinated with the O X19 serum. In particular, he obtained one variant in which the O antigen was the same as that in O X2, a strain of *B. proteus* cultivated from a case of typhus fever, and associated by agglutination with a special group of typhus cases. The change in the O antigen was demonstrated by agglutination, absorption of agglutinins and complement fixation. The results do not appear to be explicable on the assumption that the variants are simply R forms resembling those in the Salmonella group, &c.

The existence of variation due to change in the somatic antigen in the *B. proteus* group is especially interesting, since this part of the micro-organism rather than the H component is that which distinguishes the different strains of *B. proteus* which occur naturally, and especially the X strains from the rest (Weil and Felix). The H antigen is uniform throughout a large section of the *proteus* group, but is much more varied in the Salmonella group.

Correlated Variation.

The different variable characters mentioned above may vary alone without any other evident change, but often the change occurs at the same time in a group of characters. Indeed, it is fairly certain that some characters are directly interdependent, but one character may vary more prominently than another which is usually essentially linked to it, because the one is a more sensitive index of change than the other. Different groups of interrelated characters will be briefly summarized, taking first what is regarded as the most essential character. These examples will refer chiefly to bacteria in the intestinal Gram-negative family.

Group 1. Flagella ; motility ; floccular form of agglutination with serum ; presence of heat-labile antigen (100° C.) ; tendency to confluent growth on agar ; agglutination with acids at Michaelis' characteristic zones (pH 5 to 4).

Group 2. Presence of S, heat-stable antigen ; smooth, domed, translucent, non-granular colonies on agar ; formation of granular or

sandy, dense clumps with specific serum; stability of emulsions in salt solution; uniform turbidity of broth cultures; pathogenicity; prophylactic value in a vaccine.

Group 3. Characteristic R heat-stable (degraded) antigen, which is common to several allied species; colonies on agar irregular in outline and surface, coarsely granular or often opaque; making a large, often granular deposit in broth cultures with clear supernatant, often with a coherent surface film; spontaneous agglutinability in salt solution and often in low dilutions of normal serum; weak agglutinogenic activity; feeble virulence; and in some cases, low antigenic and protective power as a vaccine.

Group 4. In the case of *Pneumococcus*, (i) ready solubility in bile; and in this species and *Pneumobacillus*, the presence of a capsule; and of the specific soluble substance; type specificity; virulence; characteristic translucent, non-granular 'smooth' colonies: (ii) the absence of the soluble specific substance, and capsule; but instead the agglutinable substance and agglutinogen common to all the R or degraded forms of the species; irregularity and granularity of colony; less ready solubility in bile; sometimes growth in broth as a deposit; low virulence.

Group 5. Loss of motility and change from S to R form have been observed to be correlated, but this is rare, it has only been found to occur regularly in one strain of *B. suipestifer*, hog cholera XII.

Group 6. Agglutination by acids in Michaelis' special zone, i.e. when the critical pH is between 4 and 5, as far as has been observed is always associated with the presence of abundant flagella and flocculent agglutination with serum.

It is seen then from the few instances given above that the form of colony, or the manner of growth on agar or in broth, may be associated with special features in relation to agglutination by salt, agglutination by acid, motility, reaction to a particular antibody, agglutinin, bacteriolysin, &c., high or low pathogenicity, or solubility in bile or special chemical composition.

Dissociation.

This term was used by De Kruif (1921[2]) in describing his D (diffuse) and G (granular) forms of *B. lepisepticus* which bear considerable resemblance to the S and R forms in the motile, intestinal group of bacteria. It refers to the phenomenon by which definite species split up into a number of distinct forms, most, if not all, of which, are capable of returning to the normal without exceeding the bounds of the species.

Hadley (1927) in collecting and annotating a most valuable mass of published information on this subject including his own experiments and observations, has used the term '*dissociation*' and proposes that it shall be preferred to variation and mutation for such changes in cultures. He, at the same time, definitely attributes these phenomena to cyclogenic phases. It is by no means finally settled, however, that the so-called S

and R forms, for instance, in different groups of bacteria are all separated by the same essential changes, or are, indeed, strictly speaking, stages in a cycle. In the Pasteurella group, for instance, e.g. *avisepticus*, though both diffuse, or turbid, and clear, granular cultures have been recognized and described, the associated characters, e.g. agglutination by salt and differences in agglutinogens, have not been so regularly obtained, although loss of virulence has often been associated with a clear broth culture, as in Salmonella. Moreover, the extent and limits of variation in most cases have not yet been fully explored.

Methods of Studying Variation.

Mass Cultures.

Formerly these studies were chiefly carried out by working with mass cultures. As examples out of a large number may be cited the following observations. It was found that frequent subculture in normal broth or serum-broth increased the virulence of some cultures, e.g. streptococci, but this change was often very fugitive, the culture reverting to its previous state. Again, the growth of *B. typhosus* in dulcitol produced a comparatively stable race which rapidly fermented this alcohol, instead of requiring several days or weeks, and Twort (1907) by a similar process of ' training ' induced one strain of *B. typhosus* to ferment lactose rapidly. Ledingham (1918) reported acquisition and loss of ability to ferment isodulcitol and raffinose by *B. dysenteriæ* (Flexner), and like variation has been found to occur in some strains of *B. dysenteriæ Y* as regards cane sugar. Again, Ainley Walker (1921) and others, produced a dys-agglutinable strain of *B. typhosus* by growth in an immune serum. Sobernheim and Seligmann (1910) and Beniasch (1912) described a change in the acid and serum agglutination of *B. paratyphosus B*. On the other hand, inagglutinable cultures have often been made more readily agglutinable by frequent subculture in broth. Mass cultures also change as a whole as regards motility, rate of growth, size and appearance of the colonies, &c.

Penfold (1913[1]) found that reversion to gas production in an anaerogenic variant took place more rapidly in a large two- to three-litre flask than in a smaller volume, and argued that this was due to the large amount of growth unrestrained by crowding and by-products, since this rapid growth led to the maximum variation and the larger number of variants for selection to act on.

Revis (1911) produced a hereditary variant which did not form gas from sugars or alcohols by growing *B. coli* in mass culture in a medium containing malachite green. The whole culture or approximately the whole culture, was changed and appeared to have acquired the new character permanently.

Such mass changes in cultures only approach complete transformation in a few instances, and in many cases no persistent new variant is procured, but only irregular fluctuation in one direction or another, without any

certainty in the direction of the change or permanence of the final product. Similar attempts have been made to train mass cultures to resist phenol and other disinfectants. In these cases a slight shift in the properties of the cultures has been obtained, but has not proved progressive, and no permanently highly resistant strain has been produced.

Isolation of Variants.

By selection of single colonies. The study of variants by the selection of single colonies on solid media has very much advanced knowledge on the subject. Separate colonies picked off the same plate of a pure culture have been found to have quite distinct characters, and these differences have sometimes proved permanent.

Some variants appear never to be obtained quite pure or to become fixed. These constantly reverting variants were called by Toenniessen ' alternants ' in the case of *B. pneumoniæ* (Friedländer) and its capsulated and non-capsulated forms. On the other hand, in many cases if a variant is replated it is found that the number of variants far exceeds that of the original parent colonies, and, when one of the variant colonies has been selected and replated several times, a pure culture can be obtained, which remains pure indefinitely, or only reverts by the occasional appearance of a few atavists at long intervals. This method of selecting colonies by their appearance is a fruitful means of securing variants in many kinds of bacteria, as was shown by Baerthlein (1918), but entire dependence on the size, shape or colour of a colony is often deceptive, if this alone is taken as the criterion of variation. It is usually very important to find some other method of demonstrating the change which has taken place, or of bringing into prominence some other correlated change in character which corroborates the variation, otherwise the apparently new form is often found to have quite fugitive and unimportant characters. As an instance, may be adduced the detection of non-motile or O colonies of *B. proteus* by their isolated occurrence without any spreading film on agar. Since the film is to a considerable extent dependent on moisture of the agar, the reality of an O variant needs to be confirmed by subculture in broth, and examination for motility or by agglutination with serum to determine the absence of the H antigen.

Similar and often greater difficulties are experienced in selecting a pure rough form of *B. typhosus* or similar bacterium. The typical flat colony with irregular outline and shagreened surface usually denotes a pure R form, but this may be occasionally simulated by other colonies which are antigenically smooth. Again, the truly R form may occur in colonies differing little in appearance from the normal, except by slightly greater opacity or granularity or dullness of surface. In these cases the reality of the R change may be verified by the occurrence of spontaneous agglutination in broth culture or of an emulsion in NaCl solution. In other comparatively rare instances colonies consisting of altered R bacteria resemble the normal in all respects, and even do not readily

agglutinate in salt solution and grow with uniform turbidity in broth, but yet when tested with agglutinating serum are found to have lost their S heat-stable antigen and acquired that appropriate to the R form (Goyle, 1926, 1927 ; P. B. White, 1926).

In the case of variation in the direction of roughness the change is often more gradual than in some other kinds of variation. The culture is often found to consist of bacteria yielding uniform colonies, which, however, are not pure in their constitution, since they exhibit half-way characters, belonging partly to the S and partly to the R form. No doubt in these cases both S and R heat-stable antigen is present in each individual bacillus. The culture then is only slightly agglutinable by saline, and the colonies are usually granular but not of a typically rough aspect.

By single cell culture. Various methods have been devised for obtaining cultures from a single bacterial cell, by observing single separate bacilli under the microscope and either removing them in a capillary pipette as in Barber's technique and culturing each separately, or by marking the position of bacilli lying apart and watching their development so as to secure a colony from one bacillus only. The latter is the method usually practised, as in the various modifications of Burri's Indian Ink technique. These methods are described in Vol. IX. All of them require considerable care to be sure that the resulting colony is quite certainly derived from one cell, and in very many instances the spreading of a *uniform emulsion not containing clumps* on an agar surface is nearly as free from the same error. If, moreover, a number of colonies are selected and are found to yield similar cultures, and if, further, the spreading and selection of colonies is repeated several times, the chances that the resulting colonies are due each time to the association of the normal and the same variant are practically negligible.

Such a technique as Barber's or Burri's is needed in some cases to ensure that the initial culture from which variants are to be grown has really originated from a single bacterium. The instances in which this is required are those in which an emulsion free from clumps or cohering bacteria cannot be obtained. There are, however, cases in which the ' single cell techniques ' are especially hard to carry out.

Serious objections to the habitual use of these methods for studying variation are : (1) that the above-mentioned uncertainty as to whether colonies are derived from single cells is not entirely overcome even by the best technique ; (2) that there is great difficulty in attempting to secure rare variants by a slow technique : a variant is often only obtained after very numerous subcultures, plating, &c., which could not be carried out with sufficient repetition if cumbered by repeated ' single cell culture ', and probably every individual bacterium is not equally suited to be a starting-point for a search for variants ; (3) in many instances it is necessary to select a very large number of cells before one which is viable can be obtained, e.g. meningococci and some anaerobes ; (4) if a ' single cell '

culture is only used to obtain an initially pure culture of the parent strain, before the search for variants is begun, the method is often practicable, but subsequent prolonged culture and subculture sometimes introduce more chances of error than arise from careful and repeated plating of well-distributed emulsions. In any case, the occurrence of given variants can only be made certain by frequent repetition of the experiment and obtaining the same results.

Valuable as these special methods are for testing the correctness of the view that certain variants have descended from normal bacteria, and the reverse, and for supporting the general theoretical position, it may be stated that whenever one of the single cell techniques has been used successfully to investigate a case of variation which had previously been found by repeated and careful plating, then the origin of the variant which had previously been ascertained by ordinary technique has been confirmed. When a variant is rare, and its appearance has only been occasionally observed, a failure to obtain it when desired after the use of a special technique for isolation is not surprising, but does not necessarily negative its occurrence. It is not to be expected that every variant of importance can be subjected to this test.

Single cell culture has actually been applied to the investigation of the changes in cultures of *B. coli mutabile*, non-gas-forming variants, virulent *C. diphtheriæ* (Crowell) ; Pasteurella (de Kruif) ; S and R variants and H and O and many others.

The variants which are of most value in establishing a theoretical position are those whose occurrence can be repeatedly confirmed. When a rarely occurring variant is reported it seldom carries conviction. It is most desirable, therefore, that the conditions leading to the variant should be investigated and brought under control so that its occurrence may be observed repeatedly.

Plating from Old Broth Cultures.

The search for variants has most frequently been successful in old liquid cultures which have been kept for some weeks or months. Old cultures in ordinary broth often yield a considerable variety of colonies when plated on agar or gelatin as advocated by Baerthlein. Change from the S to the R form and from H to O can often be obtained by this means. The composition of the liquid medium may determine the kind of change which occurs. Old cultures in liquid medium containing carbohydrates or blood-serum are a prolific source of special kinds of variants. The ageing of the culture is an important factor in inducing variation, though frequent subculture in fresh medium is a better means of producing certain kinds of variants than growth in medium which is exhausted or loaded with products of growth. The optimum procedure is probably to inoculate a large bulk of liquid and then allow it to age, examining the culture from time to time at different periods from the commencement of growth.

Selection of Variant Colonies.

It is of great assistance in the detection and isolation of variants that they should be recognizable and distinct in appearance on solid media. Many characters of colonies due to the composition of the medium on which the culture is grown have been found valuable aids in the detection of variants. Colour indicators of the pH, for instance, are such useful additions for this purpose.

Sometimes the new character is not revealed by inspection of the colonies and variants have to be sought by haphazard picking off of colonies and subsequent testing for new characters in liquid or solid media, or by microscopic examination. This is a relatively slow and uncertain method, and consequently variants which declare themselves by the characters of their colonies on solid media have been most often detected and most studied.

The variant colonies may be indicated by the production of pigment, of acid or alkali, by their shape or size, or the variant may appear as an offshoot of a parent colony after the latter is some days old. Such an offshoot may be seen as a projection from the margin, or as a granular or other change in texture of the parent colony in one part or sector. Some of the best-known variants appear on the surface of old colonies as papillæ or secondary colonies. It is probable that papillæ signify the presence of a variant which is able to grow better than the parent under the conditions existing in the colony at the time of its appearance. This enhanced power of growth may be due to the variant being able to make use of some food substance not touched by the parent race, or to the ability to overcome some inhibitory factor present. Papillæ often occur without any recognized change in the bacteria of which they are composed, nevertheless, variation may have occurred, though the kind of variation is unknown. Papillæ have been studied by Massini (1907), Müller (1911), Penfold (1910, 1911), &c., in relation to variants which have acquired the power to attack a sugar present in the culture medium ; these papillæ then assume a red colour if neutral red is present.

Other papillæ, e.g. those which appear on the surface of the thin flat colonies on agar cultures of *C. diphtheriæ*, do not, as a rule, appear till the culture is a week or more old, and grow till the whole colony may be covered by the new growth. The transformed colony then appears to be composed of individuals like those constituting the other common form of this bacillus which grows in larger, domed, smooth-looking colonies.

Atkin (1923, 1925, 1926) studied similar papillæ on old colonies of *Meningococcus*, *Gonococcus* and *Pneumococcus*, which eventually replaced the parent colony and consisted of a variant which was different antigenically from the original strain, and in some instances of greatly enhanced viability. He found that their new specific agglutination properties suggested a change from one of the recognized serological types

towards another. However, in the case of these variants the original colonies were made by a mass inoculation and may have consisted of a mixed population of forms or variants.

Variants which are manifested by papillæ and similar alterations in the colony form and composition have been recently studied by Stewart (1926, 1927) in connection with his work on *B. paracoli*. He has suggested that such changes are evidence of a Mendelian segregation of characters and manner of inheritance.

There are many indicators which may be added to solid media to make variants obvious. Besides neutral red, fuchsine, methyl violet and litmus which show changes in acidity, or assist the detection of changes in the opacity or texture of a colony, there are other substances like blood or hæmoglobin which act as indicators by showing up decolorizing and lytic substances produced by the colonies. In addition to these, inhibiting agents like phenol or chloracetic acid demonstrate variants by their differential action.

The Reality of Variation.

It is believed that true hereditary variation occurs in bacteria, and that in this process the hereditary apparatus is so changed that the new characters reappear continuously in the offspring. The extent or size of the step which is taken by the variant or mutant does not constitute an essential difference, and the chief reason for not using the term mutant in the case of bacteria is that this has been given a special meaning by botanists and applied to cases of alleged changes in the germ plasm or chromosomes. As bacteria are not known to possess any mechanism resembling chromosomes the term is now generally considered inappropriate. The term saltation (Brown, 1926) is used by botanists to denote the origin of new hereditary forms without the implications contained in the word mutation as regards changes in the chromosomes or genes. It has also been applied to bacteria (Nirula, 1928).

In opposition to the claim that many described changes in character are true variations, it has been argued that such changes in cultures are merely due to selection by one means or another of the different ' pure lines ' or constituent races supposed to be always present in a culture, or on the other hand that they are due to an extraneous infection by bacteriophage or other hypothetical ultramicroscopic parasite or symbiont of the bacteria.

In answer to the first suggestion that all so-called variant cultures are merely due to the selection of pure lines, it may be pointed out that the change is often *gradual*, and cannot, therefore, be due to a selection of pre-existing units. In other cases it is certain that though the variant colony, as soon as it appears, is quite distinct from the parent type, yet the previous purification of the culture by repeated selection of unchanged colonies negatives the inclusion of a preformed variant race. In order

further to ensure the reality of variation the best-known variants have been repeatedly obtained from cultures started from single bacteria by one-cell culture techniques.

Different kinds of variants have been raised from single cell cultures of the parent strain in several species of bacteria. Müller (1911) obtained variants in respect of the fermentation of rhamnose from single cell cultures of *B. typhosus*. Penfold (1912) used single cell cultures of *B. coli mutabile* as the source from which to obtain the well-known lactose-fermenting and non-fermenting forms. De Kruif (1922) obtained his G and D forms from single cell cultures of the D form of the bacillus of rabbit septicæmia. Reimann (1925) obtained both S and degraded or R pneumococci from single cell cultures of the S form. Jordan (1926) starting from single cell cultures made by Barber's method of smooth *B. paratyphosus B* obtained the S and the R forms, and from the R obtained both R and S cultures. Weil (1923) from single cell cultures of the O form of *B. proteus X 19* obtained variants with altered O or somatic antigen. Nirula (1928) used single cell cultures for his observations on ' saltants '. Similar observations have also been made by other workers.

It is claimed by some of those who have worked with so-called pure-line cultures, obtained by microscopic technique selection of a single cell, that when this has been done any resulting variant bacteria are necessarily completely new variants or reversions, as the case may be. The alternative view, to which it is difficult to refuse acceptance, is that many single bacteria which have the predominant characters of smooth or rough, fermenting or non-fermenting forms, &c., are in reality of intermediate composition, and should not be considered definitely pure in regard to their hereditary characters, antigens and potentialities. This impurity is shown by numerous intermediate cultures which are none the less uniform in the characters of the individual bacteria of which they are composed.

The Causes of Variation.

Infection with Bacteriophage.

The view that variants may be due to infection with an ultramicroscopic virus such as the bacteriophage, is upheld by d'Herelle (1922), and is not easy to refute since there is no doubt that the phage is able to alter cultures in such a way that R or S colonies may arise, and to produce many other different forms of colony (perhaps intermediate between S and R).

How far the origin of variants may be referred to phage or similar agencies, although the presence of the bacteriophage has not been apparent in the culture, is by no means certain. Indeed, since the nature of bacteriophage as an independent ultramicroscopic organism has not been established, a transmissible metabolic abnormality like that which is held by many to be the essence of phage action might well be the cause of a number of variants. Some kinds of variation in bacteria can be produced

at will by exposing the culture to unwonted or changed conditions of temperature, food, &c. Many of these conditions have not definitely been shown to produce changes which are hereditable, but in other cases the proof is quite conclusive.

Chemical Agents: Disinfectants.

Growth of *B. proteus* or *B. paratyphosus*, &c., on 0·1 per cent. phenol-agar has been shown by Braun and Schaeffer (1919) to change the H form into the O, i.e. to suppress the H or flagellar antigen. In some cases this change persists for one or two subcultures, but as a rule the loss is very fugitive, and the H form immediately reappears on ordinary media. It is believed that a state of relative resistance to weak disinfectants can be induced by exposure to low but increasing concentrations. The variants are, as a rule, not very far removed from the normal and perhaps their appearance can best be explained by simple survival of the fittest.

Penfold (1911[2], 1913[2]) discovered that by growing *B. coli*, *B. enteritidis* (Gaertner), *B. paratyphosus B* or Morgan's bacillus No. 1 on mono-chloracetic agar 0·1 per cent., he obtained, amongst normal colonies, others which produced acid and no gas from a number of carbohydrate substances; as a rule the gas production from alcohols such as mannitol and dulcitol was not diminished. He also found that certain other related chemical substances had a similar effect. These variants were for the most part very stable, but subcultures which had reverted could also be obtained. That the variants were of the same species and had undoubtedly descended from the parent strain was shown by specific agglutination tests.

Revis (1911), by culture of *B. coli* in 0·1 per cent. malachite green, suppressed the property of forming gas both from sugars and alcohols. Variants similar in respect of sugar fermentation have been found occurring naturally, and Penfold tested the behaviour of such an anaerogenic variant, and came to the conclusion that the change was similar to that which he had produced artificially.

Food Materials: Culture Media.

Deprivation of nutriment by growing in a very weak broth (10 per cent.) leads to changes similar to those due to a weak disinfectant. The H form of *B. proteus*, *B. paratyphosus B*, &c., may be changed into the O. This again is not a permanent change as a rule.

Sugars. Cultivation on a given sugar, e.g. cane sugar, lactose, &c., of a strain or species which does not normally ferment this sugar may lead to the appearance of variants which do produce acid from it. It seems clear that this variation is in many cases due to the contact of the bacterium with the special sugar substrate towards which the change is manifested. As an instance may be cited the production by Twort of a unique race of *B. typhosus* which fermented lactose, by growing the strain in lactose for two years.

The behaviour of *B. coli mutabile* when grown in lactose media and of *B. typhosus* in dulcitol have been described above (pp. 332, 333). Very

similar phenomena have been demonstrated with regard to the fermentation of arabinose, glycerol and isodulcitol by *B. typhosus*, and of raffinose by *B. paratyphosus B* and *B. enteritidis* (Gaertner), and very rarely by *B. ærtrycke*. Also many cultures of the *B. coli* group, e.g. *B. inconstans* and *B. paracoli*, ferment saccharose slowly when unaccustomed to this sugar. In all these cases the special carbohydrate in the culture medium is not at first fermented but only after a rather prolonged period of incubation, but variants which rapidly produce acid appear as the result of the growth of the culture in contact with the carbohydrate concerned. In none of these instances is there any evidence that the variants which have acquired the new power of fermentation can arise independently of the presence of the sugar or alcohol concerned.

Sørensen (1912) recorded a case of cystitis in which the bladder was infected with gas-forming and non-gas-forming coliform bacilli at different times. Arkwright (1913) isolated on several occasions gas-forming and non-gas-forming strains of *B. lactici* from a like case. There were also intermediate forms. The extreme variants remained constant on sub-culture on ordinary media, but the non-gas-forming could be ' trained ' to produce gas by growth in sodium formate peptone water. The two forms cross-agglutinated.

Variation in the rate and intensity of acid production by variants of the same culture can often be demonstrated in the case of *B. coli*. Schierbeck (1900) by growth in milk of a streptococcus obtained variants which had a much increased capacity for producing acid.

Continuous Growth in Broth without Subculture.

The most frequent source of variants, recognized as colonies on agar, has been old cultures in broth, which, after growth for 24 or 48 hours at 37° C., have been kept for several weeks or months in capped or stoppered tubes at 37° C. or at laboratory temperature. Baerthlein (1912, 1918) made use of such cultures from which to inoculate agar plates in his search for variant colonies of *B. typhosus*, *V. choleræ*, &c. The variants obtained in this way have been very largely R or very coherent dwarf colonies, and many intermediate and less permanent kinds have been described. Weil and Felix (1917) obtained O (non-motile) variants from *B. proteus* most readily from old cultures. Balteanu found his O form of *V. choleræ* in cultures of a similar age.

The cause of the variation under such conditions has been assumed to be an accumulation of waste products, which has made adverse conditions for growth, and so given an opportunity to a better-adapted variant, or has actually caused variation by direct effect on the nutrition of some of the bacteria present. It has also been suggested that in the old cultures there is an actual deficiency in nutriment. That this latter condition is of little importance in producing R variants has been shown by Arkwright and Pitt (1929), who found that in ordinary nutrient broth diluted 1 in 5 with distilled water or salt solution an S culture of *B. typhosus* or

B. paratyphosus remained in the S condition for many months, whereas a parallel culture in ordinary undiluted broth produced R forms in 3 or 4 weeks.

Soule (1928), by plating out old broth cultures, obtained his so-called R variant of *B. subtilis*, which was distinguished by large ' medusa-head ' colonies and growth in long non-motile threads or chains. Growth in filtrates of cultures has also been found to produce variation in several instances. Rosenthal (1926) found this a very efficient method of producing an asporogenic variant of *B. anthracis*. Gee (1927), working with a sporing bacillus which he called *B. mesentericus-vulgatus*, obtained an asporogenic variant which he called the S form, by growth of the spore-former in filtrates of either form. Both the forms were motile. The R form was putrefactive and the S non-sporogenic form did not decompose proteins. Although Gee worked with cultures believed to be derived from single spores, the occurrence of such a remarkable transformation requires confirmation, and, especially, stringent proof that the original culture did not contain two different species. Such a claim recalls also the work of Schattenfroh and Grassberger (1900), Schattenfroh (1904) and Grassberger and Schattenfroh (1907) who believed that they had transformed a non-proteolytic, glycolytic bacillus like Fraenkel's, into a proteolytic strain resembling *B. sporogenes*. Their results have, however, not been accepted by most bacteriologists. It must be remembered that when a mixed culture of two species of bacteria is grown in two different media, the character of the bacterial content may be for the most part changed by the one strain or the other finding advantageous conditions for growth in the composition of the medium.

Frequent Subculture.

Frequent subculture in fresh culture medium, and the use of large vessels in which to make cultures has been found to assist and accelerate variation, especially in certain directions. Jordan (1926) was able to change an R culture of *B. paratyphosus B* grown from a single bacterium, into a culture of the S form by subculturing it twice daily in broth. Arkwright (1921) by the simpler means of daily subculture in broth obtained a similar result with cultures of *B. typhosus* and *B. paratyphosus B*, which were apparently pure R although they had not been obtained by a special ' single cell ' technique. The time required to produce the R change is very variable. Some uncontrolled factor has yet to be discovered which accounts for somewhat different results. Broth is not always of identical composition, and more important still are the different inherent tendencies of individual strains, which are often very definite.

Growth in Large Quantities of Culture Medium.

The appearance of certain variants or an increase in their number is very much promoted by growing the culture in large quantities of liquid medium. This effect was demonstrated by Penfold (1913[1]) with *B. typhosus*

grown in dulcitol-peptone water. He made a large number of experiments in order to show the effect of the size of the population on the rate of selection of new bacterial races. The strains of *B. typhosus* with which he worked fermented dulcitol only after prolonged incubation in an ordinary test-tube. Into each of 9 beakers of 5·5 cm. diameter, and 9 test-tubes of 1·0 cm. diameter, was put sufficient litmus-dulcitol-peptone-water to make a column of liquid 1·0 cm. high. The beakers received, of course, a much larger quantity of liquid, but the aeration of the contents was practically the same in each of the 18 vessels. Among the beakers acid was first indicated in 1 on the ninth day, in 2 on the thirteenth day, in 4 on the fourteenth and in 2 on the fifteenth day. In the test-tubes no acid reaction was seen up to the twenty-sixth day. Also the 18 vessels were subcultured weekly to test-tubes. After the second subculture from 4 beakers, acid was seen on the second day, from 3 on the third day, and from 2 on the fifth day. In none of the original test-tubes, nor in the subcultures from them was acid seen by the twenty-sixth day. In another experiment it was shown that, when nine tubes in three series containing 50 c.cm., 20 c.cm. and 5 c.cm. of dulcitol-peptone-water respectively, were inoculated with *B. typhosus*, the tubes with the largest quantity showed full acidity before the tubes with 20 c.cm., and these latter before the tubes with only 5 c.cm.

Penfold also found that the reversion of an anaerogenous variant of *B. coli* occurred much more rapidly in a large flask than in a test-tube. He found, too, that the size of the inoculum had much less effect than the size of the vessel on bacterial variation. Penfold concluded from his experiments that the more rapid change was due to the occurrence of a more effective selection from the larger population, and that this was mainly due to the presence of more extreme variants in the larger population, since there were more variants of all kinds.

Soule (1928) also found that interchange of his two forms of *B. subtilis* took place in either direction from his S to his R or vice versa much more rapidly in flasks of 300 c.cm. than in test-tubes, and gave a similar explanation, i.e. that the period of rapid multiplication in the larger vessels accounted for the more rapid change. Gee (1927) found that large volumes of liquid hastened the variation in his *B. mesentericus-vulgatus* cultures.

Growth in Serum.

Quite different effects are produced as a rule by growth of a bacterium in normal serum and in the serum of an animal immunized against the same strain.

Speaking generally, normal serum used as a culture medium has a similar effect to passage through a susceptible animal, and tends to preserve or increase the well-developed S somatic antigen, and to raise the virulence of the strain ; whereas growth in immune serum produces R variants, and lowers the virulence of the strain. There is some evidence

that passage through an immune animal also lowers the virulence of a strain, as in Morgenroth's experiments on 'depression immunity' (Morgenroth, Schnitzer and Berger, 1925). The observations of Zdansky (1928) on the change in type of streptococci in septicæmia are also pertinent.

Normal serum may in some cases assist the production of R forms, but this is perhaps due to unsuspected antibodies in the 'normal' serum or to other unknown factors.

Griffith (1923) found that growth of S virulent *Pneumococcus* in serum containing antibodies to the S form of the same type changed the cocci into the R form, and this was confirmed by Reimann (1925–7). Stryker (1916) had procured similar results with most cultures. Julianelle (1926) also obtained R non-specific, non-capsulated forms of the *Pneumobacillus* of Friedländer by growing the virulent capsulated form in specific antiserum of the same type.

The effect of growing a smooth strain in corresponding antiserum is well shown in the experiments of Arkwright and Pitt (1929). Smooth strains of *B. typhosus* and *B. paratyphosus A* were subcultured in different media, and the effects of these compared by subculturing and by plating out on agar at varying intervals, after growth at 37° C. in capped tubes. The different tubes contained (1) ordinary broth, (2) broth diluted 1 in 5 with distilled water or salt solution, (3) one part of normal rabbit-serum diluted with 3 parts of distilled water and 1 part of broth, (4) immune serum versus the S strain and (5) immune serum versus the R strain of the same bacterium; each of the last two tubes was diluted like the normal serum. The results of growth in several such series of tubes were fairly uniform. The tube containing immune serum versus the S form was always the first to produce R forms and these usually appeared about the tenth day. The R form was next found in the tubes containing pure broth, usually after four or more weeks. The tubes containing diluted normal serum have not produced R regularly, but only rarely a few colonies. The tubes with diluted broth and diluted R serum have usually remained free from R forms for several months. It was found that growth of *B. paratyphosus A* in antiserum to the smooth O (non-motile S) form had the same effect as growth in the smooth H (motile S) antiserum. This fact confirms the view that the change from the S to the R form only concerns the somatic and not the flagellar antigen. They found, too, that a serum versus the S form of *B. enteritidis* (Gaertner) which contained antibodies to the S somatic antigen for S *B. typhosus* was equally effective in the same direction, i.e. in turning an S into an R culture of *B. typhosus*, but that it lost this property when heated at 70° C. or absorbed with an S culture, by which means the somatic antibody was removed.

Rough cultures of the same strains as were used in the experiments with the S cultures were also grown in pure broth, diluted broth, diluted normal serum and S and R antisera. The R strain generally remained unchanged in all the tubes except that containing R serum. In these,

sometimes after several weeks, colonies which superficially resembled the S colonies were obtained. It was found, however, that these colonies, though giving more turbid and stable growth in broth, and less readily agglutinating with salt solution, were not by any means typical S forms. Sometimes they gave a little agglutination with S serum but not more than with normal and R sera. These smooth-looking colonies were, therefore, not considered to be S antigenically, but perhaps really in a more advanced state of antigenic 'degradation' than the original R. They resembled the strain of *B. typhosus* '162' which Goyle (1926) found to be S in cultural features but R antigenically. It has, however, been stated by some others who have made observations on this point, that growth of a rough strain in a serum made against this form leads to a reversion to the smooth form. These observations are of especial interest since there is still considerable doubt whether the change from R to S actually occurs in culture, if the R strain used is really pure R antigenically in the strictest sense. It seems clear that change from one form to another may occur in one of three ways: by selective survival of a few bacteria which belong to the new form but which were present in the original culture; by segregation of the S and R characters into different bacteria, these having been present all the time as a mixture of characters in the same bacilli, which were not antigenically pure; and thirdly, as the result of a more direct action of the culture medium on the bacteria.

The change from R to S by very frequent subculture effected by Jordan has been alluded to before. Dawson and Avery (1927) and Dawson (1928) reported instances of this change occurring in the case of *Pneumococcus* as the result of growth in R serum. Since Dawson and Avery themselves and others obtained reversion occasionally without the aid of R serum it is permissible to doubt whether the R cultures which they used were *antigenically* pure, although they were derived from single bacteria. Soule (1928) described interchange in both directions between two variants of *B. subtilis* as the result of growth of each form in its appropriate antiserum. He described the two forms as 'S' and 'R' on account of the appearance of the colonies. The 'S' form which was motile grew in typical smooth colonies, the 'R' grew in long chains, and was almost non-motile and formed 'medusa-head' colonies. It seems doubtful whether these forms had any true analogy with the S and R forms of *B. typhosus* or *Pneumococcus*, for though there was an antigenic difference, this may have been due to the prevalence of flagellar antigen in the S form. However, in the experiments by Arkwright and Pitt with *B. typhosus* and *B. paratyphosus A* referred to above, when the S and R cultures were motile, the motility was not altered by growth in antisera, whether these were made with motile or non-motile strains.

That growth in serum containing antibodies to a particular antigen may, in some instances, transform a culture into one in which that antigen is suppressed or altered is shown by the experiments of Scott (1926). He obtained two newly isolated strains of Salmonella—'Thompson' and

' Maidstone ', which appeared to be permanently in the ' specific ' phase of Andrewes. By growing them in homologous antiserum he induced them to change to the ' group ' phase, which then also seemed to be permanent, until by growing them in ' group ' serum he again procured growth of the ' specific ' phase. He also showed that this phenomenon occurred to a considerable extent in other types of the Salmonella group. Since the specific and group phases are only known to differ in the flagellar antigen, this antigen and antibody appear to have reacted to produce the change.

Modes of Variation.

Irreversible Variation.

The sudden appearance of a new, fully developed character in the lactose-fermenting form of *B. coli mutabile* was called ' mutation ' by Neisser and Massini because a large and apparently irreversible step was taken by this variant, and it was, therefore, supposed to resemble the mutations of De Vries. In the same way complete and persistent changes occasionally take place in other directions. The two forms, parent and offspring, are sometimes so definitely contrasted, and the change is so striking that it may be described as a segregation of characters. Complete irreversible variation occurs in other directions (Nirula). Whether such ' mutations ' are ever progressive and lead to evolution of new species, as seems *a priori* probable, is uncertain, though there is some suggestive evidence that a mutant may persist and later acquire further new characters, as, for instance, in the case of the variants of the O form of *B. proteus X*, described by Felix (1922).

Reversible Variation.

More commonly there is some tendency for variants to revert to the parent form. The variation then takes one of two main courses :

(1) *Alternation.* The new variant, which may be quite distinct, invariably reverts completely, at any rate after two or three subcultures, to the parent form. This kind of variant in *B. pneumoniæ capsulatus* was described by Toenniessen (1921) as an ' alternant ' in which he supposed that the ' gene ' for the varying character was latent, though still present in alternate generations. Such alternating forms are seen also in the specific and group phases of Salmonella cultures.

(2) *Progressive Selection.* In the commonest type of variation the variant at first appears in only a few colonies, but after selection and further plating it progressively forms a larger and larger proportion of the colonies, until it has led to a culture consisting entirely of the selected variant with no tendency to revert, or no greater tendency in the direction of the parent, than the original culture had towards the variant. The variant colonies may at first be only partially changed, and the change then becomes more and more complete as selection proceeds. This mode of change is seen in the gradual conversion of an S into an R form and often

in the acquisition of the power to ferment a special sugar. On neutral-red-agar, at first a few red and a few pink colonies are seen, later many red, and lastly all are pure red. The change may run a similar course when a strain of *B. typhosus* or *B. coli* first produces a variant whose distinctive property is that individual bacteria are longer in shape than usual. If a colony with some long forms is selected, the morphology of most of the rods may be longer than normal, but after two or three selections the culture is found to consist almost entirely of long rods and threads, and this character may persist in subculture without further selection. The same mode of change is often observed in the course of obtaining a non-motile from a motile form of *B. typhosus* or other strain of bacterium, and in many other kinds of variation. It must be supposed that in these cases each bacillus gives rise to a proportion of normal and a proportion of more or less completely changed variants, and that by the selection of the purer of the variants a culture is eventually obtained consisting of individuals which give rise to only one kind of progeny.

Change in Part of a Colony.

The first evidence of variation may be seen in an alteration in the appearance of part of a colony, especially if the culture is a few days old. These changes may be shown (1) by an opacity or granularity at one side or in one sector, as often happens in the R change. Very striking instances of variation, or ' saltation ', by sectors have been described by Nirula (1928) in *Azotobacter*, &c. ; (2) by the appearance of marginal outgrowths, which may indicate either the production of motile forms from a colony of previously non-motile units, or some other change which first begins in a similar way. According to Hadley (1927) and Stewart (1927) such marginal variation may be associated with change from the S to the R form ; (3) in other cases secondary colonies or papillæ arise on any part of the surface of a colony. The new form originating in a papilla is often distinguished when subcultured by an unusual capacity for vigorous growth and multiplication under the same conditions as the original culture. Variation by means of papillæ may concern the fermentation or other properties.

Segregation.

The phenomena of variation in bacteria, as in many other living things, may be characterized by the appearance or disappearance of one definite unit character, which may occur suddenly without change in the other units that make up the specific characters. The change may consist of the loss or gain of a character, but whether it is due to a *plus* or a *minus* change in the component hereditary factors is generally merely a matter for speculation, e.g. loss of an inhibitory factor may perhaps explain the appearance of a new fermenting capacity. Very well-marked variants which seem to be extreme representatives of some special character can often be best detected by plating very old liquid cultures. In certain strains of bacteria individual variants fermenting and not fermenting dulcitol,

lactose or raffinose have been found more distinctly differentiated in old cultures on these sugars than in young. Stewart (1926), impressed by the completeness of the change in these latter variants, has recently put forward the hypothesis that the appearance of red (fermenting) papillæ of *B. coli mutabile* and *B. paracoli* on neutral-red-sugar-agar is an instance of real Mendelian segregation. He regards the fermenting variant as recessive and due to the loss of an inhibiting factor. He thinks the papillæ themselves are the seat of ' autogamy ' and indicate a sex-like phase in the life-history, at which segregation, reduction and reassortment of genes occur, as in the higher nucleated organisms. He further believes that, in order to induce the lactose-fermenting variation of *B. coli mutabile*, the stimulus of the presence of lactose must act at the critical period at which the papillæ are being formed. The papillæ formed in the absence of lactose do not contain lactose-fermenting bacilli, though he regards the papillæ in the two cases as otherwise identical. Stewart found that if, when a papilla on ordinary agar with neutral red was in process of formation, a pit was made in the agar and a little solution of lactose put into it, then the papilla which began without lactose-fermenting properties rapidly acquired this power as the result of the stimulus of the lactose. This very interesting experiment needs to be repeated and amplified. It is not certain, however, that the papillæ are due to the same cause in the two instances. Stewart's hypothesis is complicated by the assumption that the presence of lactose and the occurrence of a sexual process must be simultaneous in order that the change in fermentative properties may be produced. The suggested scheme of Mendelian segregation is, moreover, incomplete, since his observations only record ' homozygous recessives ' but no homozygous dominants except very rarely, and enumeration of the results of segregation is impossible, since the individual bacilli cannot be distinguished, but only the colonies formed from them. It does not seem likely that the kind of segregation observed in bacterial variation is really comparable with the segregation of Mendelian characters or the separation and rearrangement of ' genes ' and ' chromosomes ' in higher plants and animals. There are frequently stages in bacterial variants when the segregation is incomplete, and half-way characters are the more common. It seems more probable that according to the hypothesis of Müller and Penfold, the papillæ are vegetative outgrowths due to the acquisition of power to ferment or otherwise to make use of a new form of nutriment, or to overcome some repressing factor. The papillæ in the absence of lactose are then due to excessive growth of certain members of the colony which are adapted by variation to some special condition present in the substrate other than lactose. It is probable that papillæ usually signify a period of stress in regard to nutrition, and if a new substance like lactose is present a variant using this is enabled to multiply rapidly.

The sudden and irreversible variations in bacteria which have been called ' mutations ' are evidence of this kind of ' segregation ', but ' mutation ' is not a suitable term, because conjugation and sexual

processes cannot be demonstrated, and are probably not involved, and the genetic constitution cannot be determined as in higher forms. The variations in bacteria probably correspond to the ' Dauermodificationen ' occurring in the vegetative stages of protozoa described by Jollos (1924). They depend on the occurrence of new characters or the unequal distribution of pre-existing characters in the offspring. Whether the change is complete or partial depends on the degree of ' segregation ' of the characters involved. The sexual processes of higher forms of life with definite nuclei are associated with true Mendelian segregation of characters, but a degree of ' segregation ' or separation also occurs during the non-sexual vegetative stages of higher plants and of protozoa as well as of bacteria.

The Nature and Limits of Variation : Dissociation and Life-Cycles.

The nature of hereditary variation in bacteria, that is to say, its true place in the life-history of a race or species cannot yet be stated with finality. Two main hypotheses are currently held to explain the observed phenomena—cyclogeny and hereditary variation.

Cyclogeny.

The varying forms described are held by those who use the term ' cyclogeny ' to consist of stages in the life-history of the species, which eventually tend to return to the starting-point. The stages are marked by very varied morphological and physiological changes. The cycles involved may be simple or multiple and may be related to each other in a complex manner. It is believed that there are special means of reproduction other than simple fission. Such are the formation of buds which break loose or the production of ' gonidia ' inside the body of the bacterium, which are extruded and then perpetuate the organism by growth and multiplication. Appearances very suggestive of this process have been seen by many observers, but most of them have reserved their judgment as to the viability of these products. In some cases a kind of conjugation or the formation of a symplasm or even a sexual phase has been described. As in higher plants, all these changes are said to take place within the boundaries of the species, and the effect of environment is to induce the onset of certain ' cyclostages ' but not to alter the hereditary factors. This scheme represents an attempt to interpret the facts by the known life-histories of higher fungi, and to avoid admitting that the fresh characters which appear in a culture are in any sense new to the species. It has been advocated by Löhnis (1922) and Mellon (1919–25) and has been described with great detail as a complete system by Enderlein (1905). It also corresponds to the conception of ' dissociation ' advocated by Hadley (1928). This author, however, does not definitely accept as proved the occurrence of conjugation or sexual processes as part of the cycles but favours their existence as probable. A kind of life-cycle in *B. radicicola*

appears to have been amply demonstrated by Thornton and Gangulee (1926) (see Vol. III). In this case the term cycle is applied to a change in the proportion of different forms in cultures in soil with special nutrient additions. Three main forms of the microbe are described, unbanded and banded rods and rounded (coccal) forms. The last, and to a less extent the first, are often flagellated and motile. When multiplication is at its height the rounded and motile forms are most numerous and the cycle consists in excess of the different forms in turn, together with varying rates of multiplication. The order in which the prevalence of the different forms occurs is fairly constant. The complete cycle has been observed twice in 24 hours. Later observation of the same culture showed that another cycle took place in about 2 to 5 more days, according to the culture medium, &c. A special feature of the multiplication recorded consists, according to the authors, in a breaking up of the banded forms into rounded forms corresponding to the bands or segments. How far these stages correspond to the motile and non-motile variants of *B. typhosus*, &c., and whether the three or more forms found in *B. radicicola* could be isolated and found to remain constant without change does not appear to have been determined. In the studies by Thornton and Gangulee the three main forms appear to have been present throughout the cycle, which consisted as far as the evidence goes only in variation in their quantitative relations.

Hereditary Variation.

The variants, though following certain lines appropriate to the ' genus ' or ' species ' are, nevertheless, to a large extent independent of each other, and might diagrammatically be arranged rather along diverging radii than on the circumference of a circle or in circles. This view does not accept as sufficient the existing evidence for a definite life-cycle or for conjugation and sexual stages, and attributes a larger share of influence to hereditary modification by the environment. The tendency of this view is to regard the pathogenic bacteria as different and simpler in their life-histories than the higher fungi, and not to accept as proved any form of conjugation, or the existence of a nucleus and a mechanism resembling chromosomes or genes. The possibility of variants leading to permanent new races or species is not excluded on this hypothesis. According to *both* views certain bacteria pass through phases peculiar to the ' species ' or ' genus ' such as endospores or capsulated forms, &c. Advocates of *both* hypotheses are prepared to accept a large number of clearly demonstrated hereditary ' forms ' or variants, which, on the one view, are regarded as stabilized ' cyclostages ', and, on the other, as variants in which the special hereditary factors have been pure from the start, or have been purified by artificial or natural selection. In *both* cases it is recognized that the environment exerts an influence ; according to the first scheme, by inducing or stimulating passage from one ' cyclostage ' to another, according to the second, by exciting a new or repressing an

old hereditary factor, and by acting in addition as a selective agent. The *range* of variation recognized by different observers of both schools has been considerable. Of recent years, since there has been a revival of the doctrine of elaborate life-cycles, those who accept the principle of cyclogeny claim to have demonstrated very diverse morphological and physiological forms. These are described not merely as dying, deformed or ill-nourished and ill-developed accidents, but as important members of the species, and of great hereditary and functional significance. In some cases the evidence brought forward by these writers (Löhnis, Mellon) is such that, if accepted in its entirety, it would suggest that bacterial forms of the most varied morphology, staining, manner of growth and functions, often appeared in the cycle of a single species which might even merge into states indistinguishable from those of the higher fungi. Such diverse and numerous phases indeed exceed the observed limits of most fungi. The wide range of a single life-history resembles that advocated by Naegeli (1877).

Most of the forms and variants which have been described can be interpreted on either hypothesis, but some of the unusual forms which have been regarded as representing a sexual or other special reproductive phase would lend support to the cyclogenic theory if their nature were accepted. The view that bacteria are very similar in structure and life-history to the higher fungi, and that a life-cycle exists, has gained some fresh supporters recently, but the argument in favour of cyclogeny seems to the present writer to rest chiefly on doubtful theoretical grounds and forced analogies. The view that bacteria, since they are unicellular and without any definite nucleus, are simple forms which can comparatively easily be modified by the environment and can pass on such changes to their offspring appears to be the most acceptable view.

Transmutation of Species.

The question of transmutation of species is subject to the same conditions, and is related to the same hypotheses as those discussed in the last section. But in addition it is liable to the further criticism that such transformations have only the support of occasional and isolated observations, which are not accepted as proved by the majority of the advocates of either cyclogeny or of the simpler hypothesis of hereditary variation. In this discussion it is assumed that the chief characteristic of a species is that it differs from its congeners by a number of hereditary and independent characters. The transmutation from one species to another whilst under observation in culture is *a priori* very improbable, since any such change means the simultaneous or rapidly successive variation in several features which then are fixed by strict and persistent selection. Unless means are taken artificially to arrange that this selection shall take place automatically, by means of the composition of the culture medium or other conditions, the survival of individuals bearing the new characters is hardly likely to occur. Intentional selection at the same time

of several independent characters by the observer is very unlikely to be successful. The ease with which a change of this kind might occur must depend to a large extent on the number of independent characters in which the one species differs from the other. If the observed differences consisted of one or two characters such as motility, and virulence for a particular animal species, as in the case of *B. pestis* and *B. pseudotuberculosis* or of *B. mallei* and *B. whitmori*, then the likelihood of transmutation must obviously be greater than in the case of such species as *B. typhosus* and *B. dysenteriæ* where the differences are at the same time cultural, morphological, pathogenic and antigenic.

The difference between naturally occurring strains or races of Salmonella which concern the existence or proportion of one or two antigens among several present are of just the same kind as those which are known to occur in the course of variation *in vitro*. That there is a difficulty or obstruction preventing one strain of Salmonella from changing into another is probably due, not so much to the observed distinguishing characters, as to the resistance to change of a number of additional unobserved differences. The probability of transmutation in any given case depends not only on the characters which have been emphasized and used for classification, but on the general tendency of the strain to remain constant or to vary.

Relation of Variation to Evolution.

The problem of the manner of evolution has not proved relatively much easier to settle in bacteria than in higher forms. It was hoped that considering the rapidity with which multiplication takes place the evolution of a species might be observed. Under the most favourable circumstances one generation of some bacteria occurs every 20 minutes, or 72 in 24 hours. With such a rapid succession of generations it might be expected that some evidence of evolution would be noticeable during the period of observation.

Perhaps the chief obstacle to the recognition of evolutionary change in bacteria when it occurs lies in the difficulty in deciding whether a newly observed variant is permanent and hereditary or merely a temporary form, which has no future before it unless it be in reversion to type. The fact that similar changes such as variation in the fermentation of a sugar, the alteration of antigenic proportions and properties, or the occurrence of R variation are not very infrequent has been used as an argument to show that they represent merely temporary or cyclical fluctuation. On the other hand, it is very likely that only when circumstances favour the persistence and survival of a given hereditary variant can it be fixed by selection, and thus make a step in the direction of progress.

What are the limits of species ? Much has been made of the supposed fact that hereditary permanent variations in bacteria are all in the direction of *loss* and not of *gain*, and that, therefore, they are non-progressive and indicate degeneration.

Two objections to this may be urged, firstly, that changes such as the acquired faculty for fermenting a sugar or the replacement of one antigen by another are *prima facie* gains and not losses, and secondly, that in such matters it is very difficult or impossible to distinguish a gain from a loss, since an apparent loss may be due to the gain of some factor inhibiting or overshadowing another and *vice versa*.

There is considerable evidence that bacteria adapted to living in the animal body, or even in blood-serum, acquire new characters of virulence and associated changes in chemical composition (antigens), and that others by training or contact acquire the property of forming acid from a sugar not usually attacked. Some of the accounts of change from saprophytes to parasites and *vice versa*, e.g. *C. hofmanni* to *C. diphtheriæ*, *B. subtilis* to *B. anthracis*, and *B. phlei* to *B. tuberculosis*, may have been based on erroneous observations, but others appear to be well founded, if not conclusively proved, e.g. from the R form of *Pneumococcus* type I to the S form of *Pneumococcus* type I or even type II, and from R *Pneumococcus* group IV to S type II (Griffith, 1928), and from non-virulent spirochætes of tap-water to virulent *Leptospira icterohæmorrhagiæ* by growth in serum (Uhlenhuth and Zuelzer, 1922).

The more common changes from H to O and from S to R forms may also be cited, though sometimes excluded as degeneration processes. The frequency of reversion in many variants may be merely due to the absence of continuous and stringent selection. When artificial selection is persistently applied a large number of permanent variants results.

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